Transiently Misacylated tRNA Is a Primer for Editing of Misactivated Adenylates by Class I Aminoacyl-tRNA Synthetases†

Brian E. Nordin and Paul Schimmel*

The Skaggs Institute for Chemical Biology and Departments of Molecular Biology and Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received June 18, 2003; Revised Manuscript Received August 22, 2003

ABSTRACT: The genetic code depends on amino acid fine structure discrimination by aminoacyl-tRNA synthetases. For isoleucyl- (IleRS) and valyl-tRNA synthetases (ValRS), reactions that hydrolyze misactivated noncognate amino acids help to achieve high accuracy in aminoacylation. Two editing pathways contribute to aminoacylation fidelity: pretransfer and post-transfer. In pretransfer editing, the misactivated amino acid is hydrolyzed as an aminoacyl adenylate, while in post-transfer editing a misacylated tRNA is deacylated. Both reactions are dependent on a tRNA cofactor and require translocation to a site located ∼30 Å from the site of amino acid activation. Using a series of 3′-end modified tRNAs that are deficient in either aminoacylation, deacylation, or both, total editing (the sum of pre- and post-transfer editing) was shown to require both aminoacylation and deacylation activities. These and additional results with IleRS are consistent with a post-transfer deacylation event initiating formation of an editing-active enzyme/tRNA complex. In this state, the primed complex processively edits misactivated valyl-adenylate via the pretransfer route. Thus, misacylated tRNA is an obligatory intermediate for editing by either pathway.

Fidelity in the transduction of biological information stored in the genetic code depends on precise molecular recognition. Aminoacyl-tRNA synthetases initiate this process through the specific attachment of amino acids to the 3′-end of tRNA (*1*−*3*). The aminoacylation of tRNA occurs in two steps. First, an amino acid and ATP are condensed to give an aminoacyl adenylate. Subsequently, the aminoacyl group is transferred to the 3′-end of the tRNA. While most aminoacyl-tRNA synthetases select their cognate amino acid with high fidelity in the first step of the reaction, both the isoleucyl- and valyl-tRNA synthetases mistakenly activate amino acids that are structurally similar to their cognate amino acids. These two enzymes achieve high fidelity in the overall aminoacylation reaction through secondary editing reactions (*4*−*6*).

Isoleucyl-tRNA synthetase (IleRS)[1] and valyl-tRNA synthetase (ValRS) are closely related, large, monomeric class I aminoacyl-tRNA synthetases. *Escherichia coli* IleRS misactivates valine, which differs from isoleucine by a single methylene unit, at a rate only 180-fold reduced from that for isoleucine (*7*). Correspondingly, *E. coli* ValRS misactivates threonine, an isostere of valine, 250-fold more slowly than it activates valine (*8*). These error rates are significantly higher than the error rates observed in the net aminoacylation reaction. Editing of misactivated amino acids occurs at two distinct points along the aminoacylation pathway. Noncognate aminoacyl adenylates can be directly hydrolyzed in what is termed pretransfer editing. Alternatively, the tRNA may be transiently misacylated and subsequently hydrolyzed in a post-transfer editing step (*9*). Both editing pathways result in the net conversion of ATP to AMP, while regenerating the free amino acid and tRNA.

IleRS and ValRS have a second active site where both hydrolytic editing reactions are catalyzed. This site is located approximately 30 Å from the site for aminoacylation, within a large insertion (termed connective polypeptide 1, CP1) into the conserved class I active site domain. The CP1 insertion from both IleRS and ValRS as an isolated polypeptide catalyzes deacylation of mischarged tRNA (*10*). The crystal structure of *Thermus thermophilus* IleRS revealed the editing site to be in a conserved, threonine-rich cleft of CP1 (*11*). Crystal soaking experiments demonstrated that this site binds valine, but sterically excludes isoleucine. Subsequent mutagenesis analysis confirmed that this site was the editing center (*11*, *12*). These findings raised the issue of how the substrates for editing might be translocated 30 Å from the site for aminoacylation to that for editing.

A possible solution to this apparent dilemma was suggested by the cocrystal structure of *Staphylococcus aureus* IleRS with its cognate tRNA (*13*). The 3′-end of the tRNA (though not completely resolved) was located in the editing domain, while the anticodon stem/loop was anchored to the C-terminal α-helical bundle that is known to be responsible for anticodon recognition. Thus, it seems likely that, following an errant aminoacylation, "flipping" of the acceptor

† This work was supported by Grant No. GM15539 from the National Institutes of Health and by a fellowship from the National Foundation for Cancer Research. B.E.N. was supported by a Howard Hughes Medical Institute Predoctoral fellowship.

* Corresponding author: Paul Schimmel; 858-784-8970 (phone); 858-784-8990 (fax); schimmel@scripps.edu (e-mail).

[1] Abbreviations: 2′-dA, 2′-deoxyadenosine; 2′-NH_2-A, 2′-amino-2′-deoxyadenosine; 2′-F-A, 2′-fluoro-2′-deoxyadenosine; 3′-dA, 3′-deoxyadenosine; 3′-NH_2-A, 3′-amino-3′-deoxyadenosine; 3′-F-A, 3′-fluoro-3′-deoxyadenosine; CP1, connective polypeptide 1, GlnRS, glutaminyl-tRNA synthetase; IleRS, isoleucyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; tRNA NTase, tRNA nucleotidyltransferase; ValRS, valyl-tRNA synthetase.

10.1021/bi035052q CCC: $25.00
Published on Web 10/18/2003

stem from the aminoacylation active site to the editing site could translocate the mischarged amino acid for editing, while the rest of the tRNA remains bound. However, the structure could not clarify how pretransfer editing could occur. Crystallographic analysis of the *T. thermophilus* ValRS/tRNA^{Val} complex revealed a nearly identical mode of tRNA binding (*14*). Additionally, a channel consisting of both protein and tRNA was proposed that could allow for the translocation of a noncognate aminoacyl adenylate. Crystal structures of the closely related leucyl-tRNA synthetase (LeuRS) revealed that the same site in CP1 can bind analogues of both pre- and post-transfer editing substrates (*15*). Thus, one site is used to hydrolyze two distinct substrates; however, mutational analysis (of IleRS) showed that distinct residues are needed for aminoacyl ester versus aminoacyl adenylate hydrolysis (*16*).

Editing via either the pretransfer or post-transfer pathway is dependent on a tRNA cofactor (*4*, *6*). Thus, in the absence of their cognate tRNAs, IleRS and ValRS activate amino acids to the corresponding adenylates, stably sequestering the adenylates within their active site. Upon addition of tRNA to a cognate aminoacyl adenylate/enzyme complex, the properly charged tRNA is generated. But addition of tRNA to a noncognate aminoacyl adenylate/enzyme complex results in rapid hydrolytic editing.

The 3′-end of tRNA is critical for editing by both enzymes (*17*−*19*). Thus, replacement of the 3′-OH group of the terminal A76 of tRNA^{Ile} yields a tRNA that is mischarged with valine and resistant to post-transfer deacylation. This result raised the possibility that the 3′-end was critical for the overall mechanism of editing. Using a variety of tRNA substrates with single atom substitutions of the ribose moiety at the terminal adenosine, we examined the role of the 3′-end of tRNA^{Ile} and tRNA^{Val} in aminoacylation, deacylation, and overall editing. Substrates were identified that were either completely or partially deficient in aminoacylation, deacylation, or total editing. Through analysis of substrates that were deficient in deacylation (post-transfer editing), for example, we attempted to decipher the separate role of the pretransfer pathway in the overall fidelity of these enzymes. These and other data gave insight into the relative contributions of the two editing pathways and to a mechanistic connection between pre- and post-transfer editing.

MATERIALS AND METHODS

Protein Expression and Purification. Overexpression of wild-type *Escherichia coli* IleRS was done in *E. coli* strain MV1184 from plasmid pKS21, which contains the gene for IleRS under control of the *lac* promoter (*20*). An editing site mutant of *E. coli* IleRS, T242P, was overexpressed in *E. coli* strain PS2766 (Δ *ilv*, Δ *ileS203::kan*$^+$) from plasmid pVDC434, a derivative of plasmid pBAD18 containing the gene for T242P IleRS under the control of an arabinose-inducible promoter (*16*). Purification of the wild-type and mutant IleRS was as described previously. IleRS concentrations were determined by active site titration (*21*).

Wild-type *E. coli* ValRS was overexpressed as a C-terminally His$_6$-tagged protein in *E. coli* strain BL21(DE3) from a pET-21b (Novagen, Madison, WI) derivative containing the gene for ValRS (*22*). Standard protocols were used for purification. The editing-deficient T222P mutant of *E. coli* ValRS was overexpressed in *E. coli* strain PS2801 (Δ *valS::kan*$^+$) from plasmid pVDC447, a pBAD18 derivative containing the gene for T222P ValRS (*23*). The protocol for purification was essentially the same as used for IleRS. ValRS concentrations were determined by the Bradford dye-binding assay.

The plasmid pET-22-CCA, which encodes a C-terminally His$_6$-tagged *E. coli* tRNA nucleotidyltransferase (tRNA NTase), was a generous gift from the laboratory of Nancy Maizels and Alan Weiner (*24*). This plasmid was transformed into *E. coli* BL21(DE3) cells, and the resulting strain was used for overexpression and purification. Protein concentration was determined by the Bradford assay.

RNA Substrates. Mature *E. coli* tRNA^{Ile} (GAU) was isolated from *E. coli* strain MV1184 containing the plasmid pES300, which allows for the IPTG-inducible overexpression of tRNA^{Ile} (*25*). Purification was as described previously (*26*). The tRNA^{Ile} could generally be isolated at a purity level of 50−70% with the remainder being other cellular tRNAs. Mature tRNA^{Val} was purchased from Sigma. The concentration of tRNA solutions was determined in aminoacylation assays from the extent of maximal charging.

tRNA 3′-End Modification Reactions. The 3′-end modified tRNAs were prepared using the tRNA NTase catalyzed pyrophosphorolysis and nucleotide exchange procedure developed in the Hecht laboratory (*27*). The *E. coli* tRNA NTase was found to be as effective as they reported with the yeast enzyme. The exchange reactions were performed in 20 mM Tris-HCl (pH 8.5), 20 mM $MgCl_2$, 1 mM sodium pyrophosphate, 6 mM ATP (or analogue), 15 μM tRNA, and 5 μM tRNA NTase. The 3′-deoxy-ATP (3′-dA) was purchased from Sigma (St. Louis, MO) and 2′-deoxy-ATP (2′-dA) was from Pharmacia (Piscataway, NJ). The 2′-fluoro-2′-deoxy- (2′-F-A) and 3′-fluoro-3′-deoxy-ATP (3′-F-A) and 2′-amino-2′-deoxy- (2′-NH_2-A) and 3′-deoxy-3′-amino-ATP (3′-NH_2-A) were purchased from Purimex (Göttingen, Germany). In all assays where A76 tRNA^{Ile} is reported as the substrate, the tRNA was prepared by the tRNA NTase nucleotide exchange procedure using ATP (as opposed to an analogue) to regenerate the wild-type 3′-end. The reactions were allowed to proceed for 4 h at 37 °C. The products were purified by ethanol precipitation, resuspension, and then electrophoresis on a 10% polyacrylamide, 8 M urea gel. Final products were generally found to be between 98 and 99% modified with an ATP analogue, as judged by aminoacylation assays. Concentrations of 3′-end modified tRNAs were determined (where possible) from their plateau levels of aminoacylation with cognate amino acid.

Aminoacylation Assays. Aminoacylation of tRNA substrates was followed by measuring the trichloroacetic acid precipitable radioactivity produced by the attachment of [^{3}H] amino acid to tRNA (*28*). Assays were performed at room temperature in 20 mM HEPES (pH 7.5), 150 mM NH_4Cl, 100 μM EDTA, 10 mM $MgCl_2$, 2 mM ATP, and 10 nM inorganic pyrophosphatase. Assays of IleRS aminoacylation activity used 500 nM enzyme, 2 μM tRNA, and 15 μM of either [^{3}H] isoleucine (1.67 mCi/μmol) or [^{3}H] valine (1.65 mCi/μmol). Assays of ValRS were performed with 1 μM enzyme, 5 μM tRNA, and 15 μM of either [^{3}H] valine (1.65 mCi/μmol) or [^{3}H] threonine (1.65 mCi/μmol). Stoichiometric mischarging of 2′-NH_2-A76 tRNA^{Val} was accomplished with 5 μM ValRS and 1 μM tRNA. For all tRNA 3′-end

variants, a range of assay conditions were examined. As the intent of the aminoacylation assays was primarily to characterize whether a given tRNA could be aminoacylated or not, the reported conditions were selected empirically to reach rapidly the maximal extent of aminoacylation.

Mischarging Reactions. Mischarged $tRNA^{Ile}$ and its respective 3′-end variants were produced using the editing-deficient T242P IleRS as previously described (*29*). Enzyme (20 μM) was in excess of tRNA (5 μM) and reactions were generally incubated for 30 min. The mischarging of 2′-NH_2-A76 $tRNA^{Ile}$ progressed more slowly, and, therefore, the reaction was allowed to proceed for 60 min. The analogous editing mutant of ValRS, T222P, was used for mischarging $tRNA^{Val}$ and its variants with threonine. Reaction conditions and incubation lengths were described previously (*29*). The 2′-NH_2-A76 $tRNA^{Val}$ charging (and mischarging) activity was significantly reduced relative to that of other 3′-end variants, such that a 6-h incubation was necessary to give high yields of Thr-2′-NH_2-A76 $tRNA^{Val}$.

Deacylation Assays. Deacylation was followed by measuring the loss of TCA-precipitatable [^{3}H] from [^{3}H]aa-tRNA. Deacylation of mischarged $tRNA^{Ile}$ was at room temperature in 150 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, using 1 μM mischarged tRNA and 50 nM IleRS. Identical conditions were used for determining ValRS deacylation except that the concentration of ValRS was 10 nM.

Editing Assays. The tRNA-dependent hydrolysis of misactivated amino acids was measured by following the consumption [γ-^{32}P]ATP as described in detail elsewhere (*30*). Assays of IleRS editing activity were done with 1 mM valine, 1 μM enzyme, and 5 μM $tRNA^{Ile}$ or 3′-end variant. ValRS editing activity was measured using 1 mM threonine, 1 μM enzyme, and 5 μM $tRNA^{Val}$ or 3′-end variant. Higher enzyme (5 μM) and tRNA (15 μM) concentrations were required to observe editing stimulated by 3′-F-A76 $tRNA^{Val}$. This assay measures ATP consumption that occurs from either pre- or post-transfer editing. All assays were corrected for a very small background rate of ATP consumption in the absence of tRNA.

RESULTS

The 3′-End of $tRNA^{Ile}$ Is Critical for Fidelity in Aminoacylation. To investigate how $tRNA^{Ile}$ controls the partitioning between two editing pathways, many 3′-end variants of $tRNA^{Ile}$ were characterized for both aminoacylation and deacylation. Those activities were then related to the overall editing activities. Previous studies showed that IleRS initially aminoacylates the 2′-OH of $tRNA^{Ile}$, so that tRNAs lacking an appropriate nucleophile at the 2′-position are not aminoacylated (*31*). Accordingly, 2′-dA76 $tRNA^{Ile}$ was not charged by IleRS (Figure 1A). Substrates with an intact 2′-OH (A76 $tRNA^{Ile}$, 3′-dA76 $tRNA^{Ile}$, and 3′-NH_2-A76 $tRNA^{Ile}$) were all rapidly aminoacylated by IleRS. In contrast to 2′-dA76 $tRNA^{Ile}$, 2′-NH_2-A76 $tRNA^{Ile}$ was quantitatively charged with isoleucine at a rate somewhat reduced from that of "wild-type" A76 $tRNA^{Ile}$ (Figure 1A). Despite the modest rate reduction, the charging of 2′-NH_2-A76 $tRNA^{Ile}$ is far too rapid (in comparison to the complete lack of charging of 2′-dA76 $tRNA^{Ile}$) to represent charging of the 3′-OH. Thus, IleRS directly charges the 2′-NH_2 group to give an amide-linked amino acid moiety.

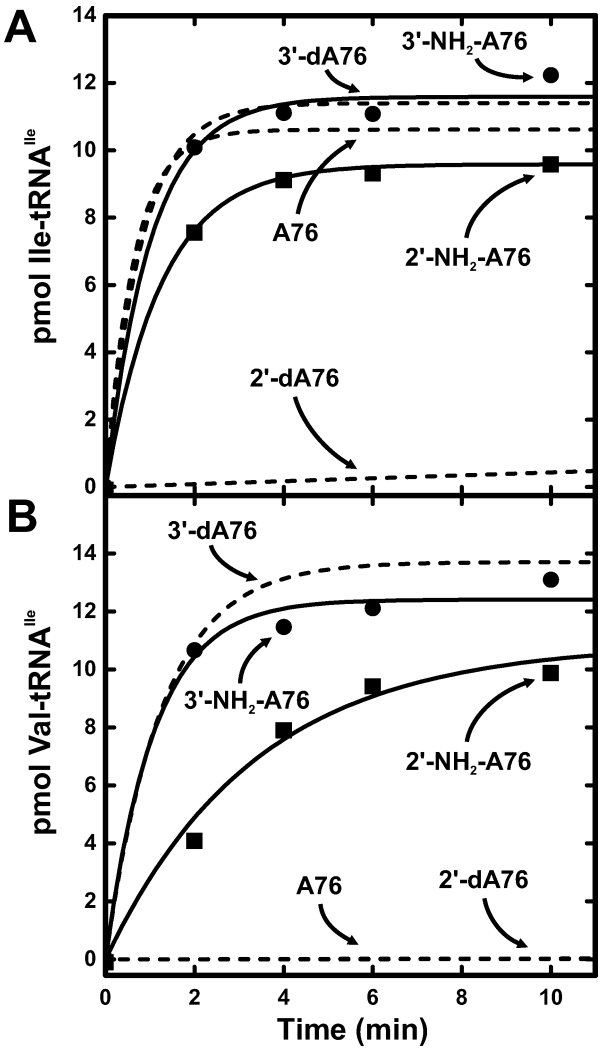

FIGURE 1: Aminoacylation of $tRNA^{Ile}$ 3′-end variants. (A) Charging with isoleucine. With the exception of the 2′-dA76 $tRNA^{Ile}$, all 3′-end variants are charged to a level equivalent to that of A76 $tRNA^{Ile}$. Even the more slowly charged 2′-NH_2-A76 $tRNA^{Ile}$ is completely charged. (B) Mischarging with valine. IleRS does not mischarge A76 $tRNA^{Ile}$. The two tRNAs containing amino substitutions are completely mischarged with valine, as is 3′-dA76 $tRNA^{Ile}$. (Dashed lines represent results reported in ref *29*.)

As expected, 3′-dA76 $tRNA^{Ile}$ was completely mischarged with valine by IleRS (Figure 1B). Also consistent with early work (*32*), we observed complete mischarging of 3′-NH_2-A76 $tRNA^{Ile}$ (Figure 1B). Although 2′-NH_2-A76 $tRNA^{Ile}$ has an intact 3′-OH, the amino group at the 2′-position disrupts both pre- and post-transfer editing. Direct charging of the 2′-NH_2 group with valine by IleRS led to complete mischarging of this substrate (Figure 1B). To contrast, neither A76 $tRNA^{Ile}$ nor 2′-dA76 $tRNA^{Ile}$ were mischarged to any detectable degree (Figure 1B).

(The results demonstrated the integrity of the 3′-ends of the substrates used throughout the current study. As seen in Figure 1A,B, mischarging plateaus for 3′-dA76, 2′-NH_2-A76, and 3′-NH_2-A76 $tRNA^{Ile}$ are identical to the respective plateaus for aminoacylation with isoleucine. Thus, the nucleotide exchange reactions successfully converted nearly 100% of the 3′-ends to the desired modified adenosine. The complete lack of aminoacylation of 2′-dA76 $tRNA^{Ile}$ also showed that it has entirely 2′-dA76 at its 3′-end.)

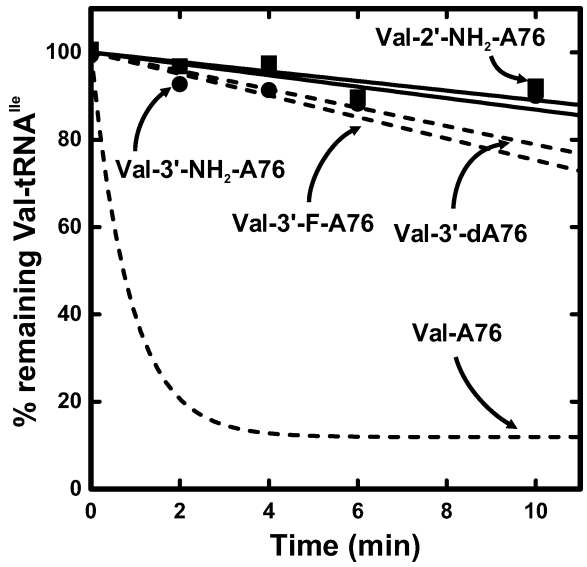

FIGURE 2: Deacylation of mischarged tRNA^{Ile} variants. Although IleRS rapidly deacylates mischarged A76 tRNA^{Ile}, all the 3′-end modified tRNAs are resistant to deacylation. The 2′- and 3′-NH_2-containing tRNAs form amide bonds upon aminoacylation. The esterase activity of the editing site is unable to hydrolyze the stronger amide bonds. Because deacylation normally occurs from the 3′-OH, tRNAs with a 3′-hydrogen or fluorine substitution (3′-dA76 and 3′-F-A76 tRNA^{Ile}) are also inactive for deacylation. (Dashed lines represent results reported in ref *29*.)

Post-Transfer Editing by IleRS. Of the two editing pathways, post-transfer editing is the only one amenable to direct measurement. Exogenously mischarged tRNA^{Ile} is easily isolated and assayed for deacylation activity in the absence of AMP or other products of aminoacylation. (The absence of AMP ensures that deacylation does not occur via the reversal of the aminoacyl transfer step.) Deacylation is sensitive to subtle changes in both the aminoacyl side chain and 3′-end of the tRNA. For example, Val-tRNA^{Ile} is rapidly hydrolyzed, while the hydrolysis of Ile-tRNA^{Ile} proceeds slowly. A mischarged aminoacyl group is only properly oriented for deacylation within the editing active site while esterified to the 3′-OH (*29*). Thus, mischarged tRNAs lacking a 3′-OH are resistant to deacylation. Following aminoacylation on the 2′-OH, the 3′-*O*-aminoacyl species can arise rapidly through a facile 2′- to 3′-transacylation (*33*).

Figure 2 displays the activity of various 3′-end variants of tRNA^{Ile} in post-transfer editing. Whereas a mischarged A76 tRNA^{Ile} is immediately deacylated, all mischarged tRNA substrates with modified 3′-ends are resistant to deacylation. The Val-3′-dA76 and Val-3′-F-A76 tRNA^{Ile} have a fixed 2′-*O*-aminoacyl group that prevents proper positioning of the scissile ester bond within the editing site. Valine is attached to the two amino-containing tRNAs through an amide linkage. (The 2′-NH_2-A76 tRNA^{Ile} is directly valylated on its 2′-NH_2 group, while an irreversible 2′- to 3′-transacylation generates an amide-linked valyl group on the 3′-NH_2 group of 3′-NH_2-A76 tRNA^{Ile}, after aminoacylation on the 2′-OH (*34*).) The editing site, evolved to hydrolyze ester bonds, is unable to hydrolyze the more stable amide linkages.

Overall Editing by IleRS. The combined activity of pre- and post-transfer editing can be followed by measuring the tRNA-dependent ATPase activity in the presence of valine. This assay potentially offers a unique way to investigate pretransfer editing. By assaying the overall ATPase activity

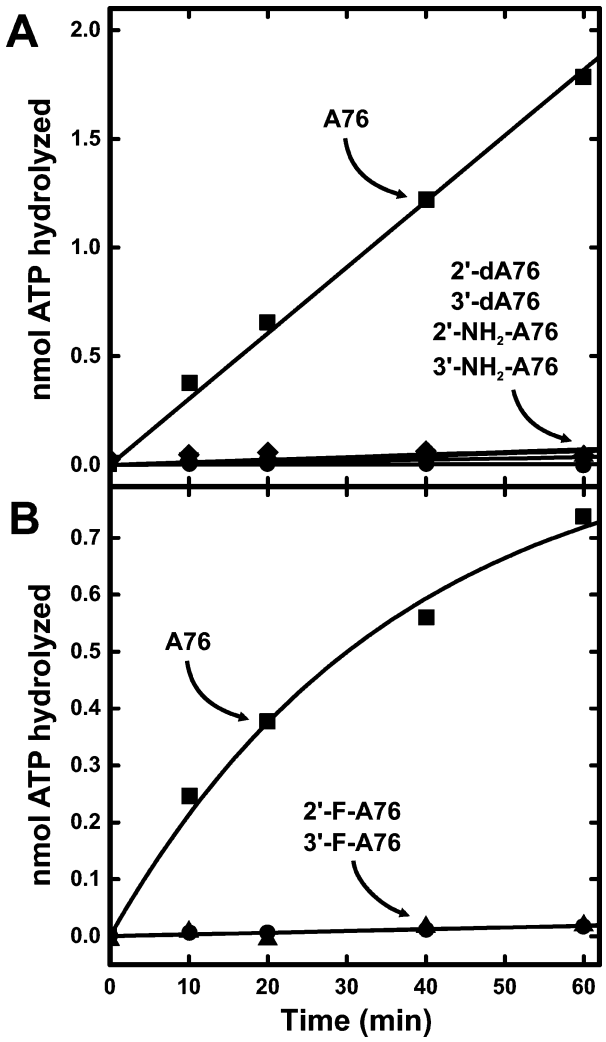

FIGURE 3: IleRS/tRNA^{Ile}-dependent editing of misactivated valine. (A, B) In the presence of valine, ATP, and tRNA^{Ile}, IleRS acts as an ATPase through the futile cycle of misactivating valine and Val-AMP or Val-tRNA^{Ile} (In both cases, ATP is consumed). Only A76 tRNA^{Ile}, which is active in both aminoacylation and deacylation, stimulates editing of misactivated valine. Neither the tRNAs that are resistant to deacylation (3′-dA76, 2′-NH_2-A76, 3′-NH_2-A76, and 3′-F-A76 tRNA^{Ile}), nor the tRNAs that cannot be aminoacylated (2′-dA76 and 2′-F-A76 tRNA^{Ile}) catalyze any editing of misactivated valine. (Panels A and B contain data from distinct protein and RNA preparations.)

using a tRNA cofactor shown to be inactive in post-transfer editing the contribution of pretransfer can in principle be estimated. Figure 3A,B shows that only A76 tRNA^{Ile} is effective in stimulating ATPase activity in the presence of valine. Substrates that were aminoacylated but resistant to deacylation, such as 3′-dA76, 3′-F-A76, 2′-NH_2-A76, and 3′-NH_2-A76 tRNA^{Ile}, showed no ATPase activity. (Early work by others reported significant ATP hydrolysis stimulated by tRNAs such as 3′-dA76 tRNA^{Ile} and 3′-NH_2-A76 tRNA^{Ile} (*35*).) In our experiments, no such hydrolysis was observed. Additionally, 2′-dA76 and 2′-F-A76 tRNA^{Ile}, which are unable to be aminoacylated by IleRS, were both inactive in ATP hydrolysis.

The lack of ATPase activity stimulated by these modified tRNAs does not arise from the lack of binding to IleRS. Those tRNAs that are mischarged with valine must bind productively. Both 2′-dA76 and 2′-F-A76 tRNA^{Ile} inhibited aminoacylation and editing stimulated by A76 tRNA^{Ile} (data

not shown). Variability in the completeness of the tRNA NTase exchange reactions for some 3′-end modified tRNA preparations also provided evidence that only A76 $tRNA^{Ile}$ is capable of stimulating editing (see Materials and Methods). For example, some preparations of 2′-dA76 or 2′-F-A76 $tRNA^{Ile}$ retained 5−10% of the original aminoacylation activity with isoleucine. This residual activity was presumed to be the result of A76-containing $tRNA^{Ile}$ remaining in the preparations due to incomplete exchange. Such preparations also retained 5−10% of the original overall editing activity. Treatment of these tRNA samples with sodium periodate followed by lysine (to destroy any remaining A76-containing $tRNA^{Ile}$) yielded tRNA preparations devoid of both aminoacylation and editing activity (data not shown). Thus, a *cis*-diol at the 3′-end of the tRNA is strongly correlated with ATPase activity.

ValRS Mischarges tRNAs with Altered 3′-Ends. To compare the editing of misactivated threonine by the closely related ValRS, we characterized a number of $tRNA^{Val}$ 3′-end variants in the same battery of assays used to test 3′-end variants of $tRNA^{Ile}$. Like IleRS, ValRS uses the 2′-OH of its cognate tRNA as the initial site of aminoacylation (*31*). Correspondingly, no aminoacylation of 2′-dA76 $tRNA^{Val}$ was observed (Figure 4A). Both 3′-dA76 and 3′-NH_2-A76 $tRNA^{Val}$ were aminoacylated rapidly by ValRS, as anticipated (Figure 4A). These two substrates were also mischarged by ValRS. However, only mischarging of 3′-NH_2-A76 $tRNA^{Val}$ proceeded to completion under the conditions of Figure 4A,B (5 μM tRNA, 1 μM ValRS). As seen previously, A76 $tRNA^{Val}$ and 2′-dA76 $tRNA^{Val}$ were not mischarged at all (Figure 4B).

Under the conditions of Figure 4A, the aminoacylation of 2′-NH_2-A76 was not complete within 10 min. The 2′-NH_2-A76 substitution causes a more significant loss of aminoacylation with ValRS, as compared to IleRS. However, under conditions of a higher ValRS concentration (5 μM ValRS, 1 μM tRNA), 2′-NH_2-A76 $tRNA^{Val}$ was effectively and completely aminoacylated by ValRS (Figure 4C). Considering the complete lack of charging 2′-dA76 $tRNA^{Val}$, the charging of 2′-NH_2-A76 $tRNA^{Val}$ must be due to direct charging of the 2′-amino group. The direct formation of an amide linked amino acid blocked editing and, as seen in Figure 4C, ValRS quantitatively mischarged 2′-NH_2-A76 $tRNA^{Val}$ with threonine. Under conditions of a high ValRS/$tRNA^{Val}$ ratio, mischarging of 3′-dA76 $tRNA^{Val}$ proceeds to completion as well (*29*). Cumulatively, the data of Figure 4 demonstrate that the 3′-end modified $tRNA^{Val}$ variants used in this work have homogeneous 3′-ends.

Post-Transfer Editing by ValRS. ValRS was active in post-transfer editing with a broader range of substrates than was IleRS. Previous work demonstrated that ValRS does not require an intact 3′-OH to hydrolyze mischarged tRNA (*19*, *29*). ValRS deacylates both Thr-3′-dA76 $tRNA^{Val}$ and Thr-3′-F-A76 $tRNA^{Val}$ with rates reduced 10- and 100-fold, respectively, relative to the deacylation rate of Thr-A76 $tRNA^{Val}$ (*29*). Figure 5 compares the deacylation activity of Thr-A76 $tRNA^{Val}$ and Thr-3′-dA76 $tRNA^{Val}$ to the two NH_2-group-containing tRNAs. The threonyl moieties of both Thr-2′-NH_2-A76 $tRNA^{Val}$ and Thr-3′-NH_2-A76 $tRNA^{Val}$ are bound to the tRNA through fixed amide linkages. ValRS was unable to hydrolyze these more stable linkages.

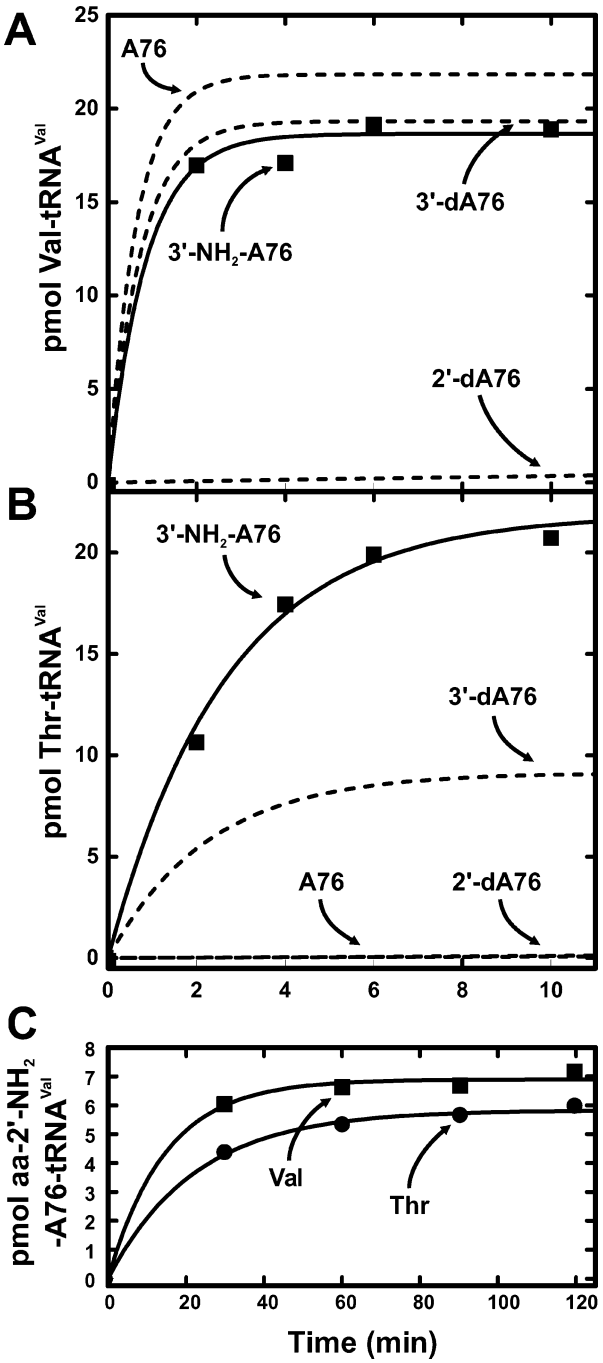

FIGURE 4: Aminoacylation of $tRNA^{Val}$ 3′-end variants. (A) Aminoacylation with valine. ValRS efficiently aminoacylates tRNAs with an intact 2′-OH. Like IleRS, it is unable to aminoacylate a 2′-dA76 $tRNA^{Val}$. Under these conditions, aminoacylation of 2′-NH_2-A76 $tRNA^{Val}$ is slow and not complete within 10 min (data not shown). (B) Aminoacylation with threonine. Both 3′-NH_2-A76 and 3′-dA76 $tRNA^{Val}$ are mischarged with threonine by ValRS. While 3′-dA76 is only mischarged to approximately 50% completion, 3′-NH_2-A76 $tRNA^{Val}$ is completely mischarged under these conditions. ValRS does not mischarge A76 $tRNA^{Val}$ or 2′-dA76 $tRNA^{Val}$ to any extent. (C) Mischarging of 2′-NH_2-A76 $tRNA^{Val}$ under high enzyme conditions. ValRS directly charges the 2′-NH_2 group of 2′-NH_2-A76 $tRNA^{Val}$, though the rate is significantly reduced from that of charging A76 $tRNA^{Val}$. In time the level of mischarging with threonine is nearly equivalent to the level of charging with valine. (Dashed lines represent results reported in ref *29*.)

Overall Editing by ValRS. ValRS has a potent ATPase activity in the presence of $tRNA^{Val}$ and threonine (Figure

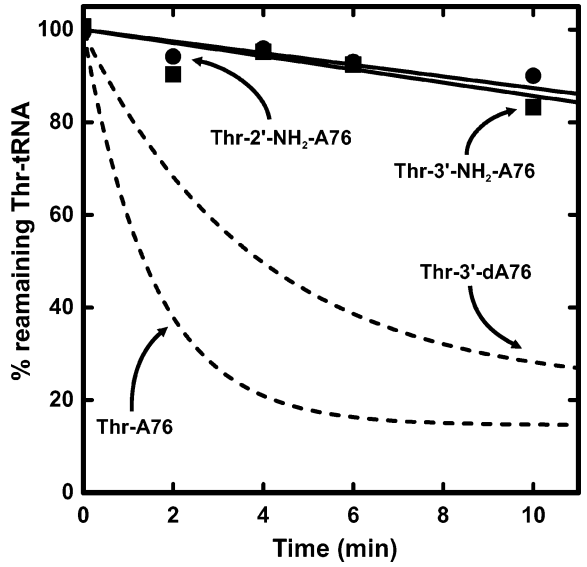

FIGURE 5: Deacylation of mischarged $tRNA^{Val}$ variants. ValRS rapidly deacylates mischarged A76 $tRNA^{Val}$. In contrast to IleRS, ValRS has a potent deacylation activity toward a mischarged 3′-dA76 $tRNA^{Val}$. Both of the amino-containing tRNAs, which form amide bonds to the threonyl moiety, are resistant to deacylation.

6A). ATPase activity could arise through either hydrolysis of threonyl-adenylate or of transiently mischarged $tRNA^{Val}$. Whereas under the conditions of Figure 6A,B (5 μM tRNA, 1 μM ValRS), 3′-dA76 $tRNA^{Val}$ clearly displayed ATPase activity, the 3′-F-A76 $tRNA^{Val}$ was essentially inactive (Figure 6B). The rate of overall editing stimulated by 3′-dA76 $tRNA^{Val}$ was approximately 10-fold reduced from that of A76 $tRNA^{Val}$. Interestingly, roughly the same reduction in rate was shown by 3′-dA76 $tRNA^{Val}$ in deacylation relative to A76 $tRNA^{Val}$. Like IleRS, those tRNAs that are resistant to deacylation (2′-NH_2-A76 $tRNA^{Val}$ and 3′-NH_2-A76 $tRNA^{Val}$) were inactive in overall editing assays (Figure 6A). Likewise, those tRNAs that cannot be aminoacylated (2′-dA76 $tRNA^{Val}$ and 2′-F-A76 $tRNA^{Val}$) were also devoid of overall editing (Figure 6A,B).

Because only tRNAs capable of stimulating editing by ValRS were also substrates for deacylation by ValRS, a closer look was taken at 3′-F-A76 $tRNA^{Val}$. This tRNA is a substrate for deacylation, although its activity is reduced 100-fold relative to A76 $tRNA^{Val}$. By increasing both the tRNA and ValRS concentrations (15 μM tRNA, 5 μM ValRS), editing of misactivated threonine stimulated by 3′-F-A76 $tRNA^{Val}$ was observed (Figure 6C). While the magnitude of the rate differences in ATP hydrolysis between A76, 3′-dA76 and 3′-F-A76 $tRNA^{Val}$ was less than for their rate differences in deacylation, their relative rank in activity is the same. (Likely, the rate of ATP hydrolysis with A76 $tRNA^{Val}$ was at its limit under these conditions.) However, even in the presence of elevated concentrations, 2′-NH_2-A76 $tRNA^{Val}$ was unable to trigger editing (Figure 6C). Thus, as for IleRS, only tRNAs that participate in both aminoacylation and deacylation can stimulate editing of misactivated threonine by ValRS.

DISCUSSION

Because both pre- and post-transfer editing require the presence of cognate tRNA, direct analysis of the pretransfer pathway is precluded. Recently, we reported the results of a

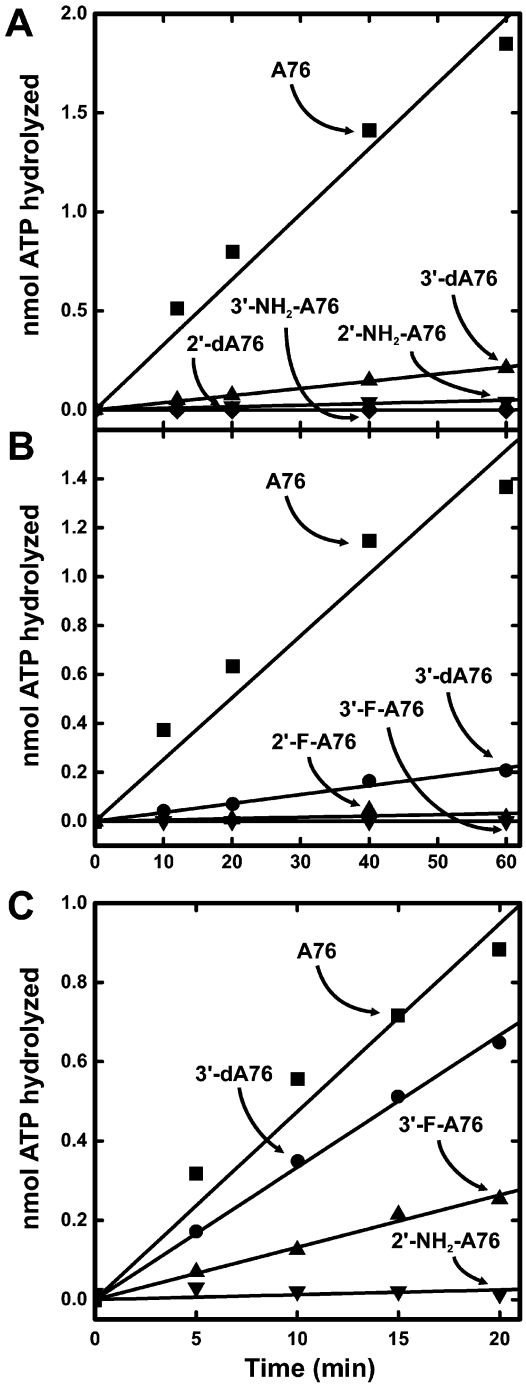

FIGURE 6: ValRS catalyzed editing of misactivated threonine. (A) ValRS editing with 5 μM tRNA, 1 μM ValRS. Both A76 and 3′-dA76 $tRNA^{Val}$ are active in the editing of misactivated threonine, though 3′-dA76 $tRNA^{Val}$ shows an approximate 10-fold reduction in rate. Both of these tRNAs are active in aminoacylation and deacylation (Figures 4 and 5). In contrast, the 2′-dA76 $tRNA^{Val}$, which cannot be aminoacylated, and the 2′- and 3′-NH_2-A76 $tRNA^{Val}$s, which cannot be deacylated, are unable to catalyze the overall editing of misactivated threonine. (B) Editing as in (A). Under these conditions, there is no apparent editing stimulated by either 2′-F-A76 or 3′-F-A76 $tRNA^{Val}$. While 3′-F-A76 $tRNA^{Val}$ can be both aminoacylated and deacylated, its deacylation rate is around 100-fold reduced from that of A76 $tRNA^{Val}$. The 2′-F-A76 $tRNA^{Val}$ is never aminoacylated. (C) Editing under high ValRS/$tRNA^{Val}$ conditions (15 μM tRNA, 5 μM ValRS). Here, the editing stimulated by 3′-F-A76 $tRNA^{Val}$ is well above background levels. Even at these elevated concentrations, a tRNA that cannot support both aminoacylation and deacylation (2′-NH_2-A76 $tRNA^{Val}$) has no editing activity.

focused investigation of the post-transfer editing reaction using a variety of 3′-end modified tRNAs (*29*). To provide more insight into the relative importance of the two editing pathways, we report here on investigations of earlier and additional 3′-end modified tRNAs (amino-containing derivatives) with assays that measure post-transfer and, separately, the combination of post-transfer and pretransfer editing (total editing). The mechanism by which a cognate tRNA can trigger translocation of a noncognate aminoacyl adenylate 30 Å across the surface of the enzyme during pretransfer editing is not well understood. Pretransfer editing was first suggested by Fersht in his analysis of the kinetic parameters involved in the editing of valine by IleRS (*36*). Fersht's kinetic data is consistent with the majority of editing (by IleRS) proceeding through the pretransfer route. A mutation (T243R) in the editing site of IleRS was recently described that lowers the overall editing rate, yet does not affect aminoacylation, deacylation, or translocation (*16*). The reduced editing rate is thought to be due to a defect specifically in pretransfer editing.

Post-transfer editing requires first aminoacylation and then deacylation of tRNA. Thus, careful testing of $tRNA^{Ile}$ 3′-end variants for aminoacylation and deacylation activity allowed for the categorization of tRNAs as those that support post-transfer editing and those that do not. When those tRNAs that were completely inactive in either aminoacylation or deacylation were evaluated in ATPase assays, no activity was detected. Wild-type A76 $tRNA^{Ile}$, the only tRNA active in post-transfer editing, was also the only tRNA that could stimulate ATPase activity. This striking correlation suggests that transiently misacylated tRNA is a crucial intermediate in the editing process.

The correlation between a tRNA cofactor that is active in both aminoacylation and deacylation with a tRNA that supports ATPase activity also held true for all $tRNA^{Val}$ 3′-end variants tested. Accordingly, a recent study of base substitutions (i.e., A to C) at position A76 of $tRNA^{Val}$ showed that disruption of deacylation always resulted in loss of ATPase activity (*37*). Thus, the requirement for a transiently misacylated tRNA during editing is conserved between IleRS and ValRS. In contrast to IleRS, Fersht found that post-transfer hydrolysis of misacylated $tRNA^{Val}$ by ValRS was rapid enough to account for the majority of editing (*6*). The relative activities of $tRNA^{Val}$ 3′-end variants in deacylation and overall editing found here further strengthen the link between post-transfer editing and ATPase activity for ValRS. In both deacylation and ATPase assays, 3′-dA76 $tRNA^{Val}$ showed about a 10-fold rate decrease relative to A76 $tRNA^{Val}$. A 3′-F-A76 $tRNA^{Val}$ was the least active in both assays. Thus, the rate of deacylation directly affects the rate of total editing.

The requirement of post-transfer editing for ATPase activity has held true when studying mutants of IleRS. For example, mutants such as T242P or D342A, which are unable to deacylate mischarged tRNA, showed no ATPase activity. The recent characterization of D342A IleRS led to the editing model shown in Figure 7A (*38*). This model, based on results with the single mutant, highlights the critical role that transiently misacylated $tRNA^{Ile}$ plays during editing. Data presented here with the 3′-end variants of the tRNA cofactor strongly support this model. Following misactivation of valine and binding of $tRNA^{Ile}$, misacylation is obligatory. Initially, there is no pretransfer editing. The Val-$tRNA^{Ile}$/enzyme complex then undergoes a conformational change, translocating the mischarged amino acid to the editing site, where it is immediately hydrolyzed. [Without hydrolysis, the "editing active" conformation (vida infra, lighter gray shading in Figure 7A) needed for pretransfer editing cannot be obtained, explaining why deacylation resistant tRNAs, such as 2′- and 3′-NH_2-A76 and 3′-dA76 $tRNA^{Ile}$, are completely inactive in editing.] Thus, after the initial post-transfer editing step, the tRNA remains bound to the enzyme in an "editing active" conformation. Possibly, this conformation resembles the conformation observed in the cocrystal structure of IleRS and $tRNA^{Ile}$, where the uncharged 3′-end of the tRNA is bound in the CP1 domain (*11*). Interestingly, both the IleRS/$tRNA^{Ile}$ complex and the ValRS/$tRNA^{Val}$ complex (*14*) assumed this conformation, suggesting that the editing active complex is thermodynamically stable (Figure 7B, left, middle, respectively). However, the "editing active" complex would presumably have the 3′-end of the tRNA in a different orientation than observed in the structure of LeuRS complexed with an analogue of mischarged tRNA, as it would block binding of the adenylate to the editing site (*15*). In this state, the complex is primed for pretransfer editing should another misactivation event occur. Likely, the RNA−protein complex only returns to an aminoacylation active state (darker gray shading) upon activation of a cognate amino acid. The aminoacylation active state conformation may resemble the conformation of GlnRS bound to $tRNA^{Gln}$ in which the 3′-end of the tRNA assumes a hairpinned conformation to enter the active site (Figure 7B, right) (*39*). While in the editing active state, the tRNA may form one wall of a channel that helps direct the noncognate aminoacyl-adenylate translocate to the editing site (*14*).

This post-transfer initiated pretransfer model explains both the complete loss of overall editing observed with $tRNA^{Ile}$ 3′-end variants and the relevance of pre-transfer editing as seen in the studies of T243R IleRS. Other potential models fail to account for all available data on the system. For example, it could be argued that pretransfer editing activity is dependent on the 3′-end of the $tRNA^{Ile}$ and that the modifications have disrupted a critical RNA−protein interaction involved in pretransfer editing. Yet, as the variants are all single atom substitutions, the set of noncovalent RNA−protein interactions that can be made should be virtually identical to the ones made between IleRS and A76 $tRNA^{Ile}$. Any of the interactions that are disrupted in a given $tRNA^{Ile}$ 3′-end variant are left intact in other 3′-end variants tested during this study. As all of the variants lacked pretransfer editing activity, no single interaction could be pinpointed as critical for pretransfer editing. While it remains possible that a specific combination of interactions between IleRS and the *cis*-diol of A76 $tRNA^{Ile}$ stimulates pretransfer editing, it seems unlikely because the combination of interactions made by either 2′-NH_2-A76 or 3′-NH_2-A76 $tRNA^{Ile}$ is nearly identical to those made by A76 $tRNA^{Ile}$. Additionally, these $tRNA^{Ile}$ 3′-end variants do not display any partial activity, which might be expected due to the loss of a single hydrogen bond.

For editing by IleRS under in vitro conditions, pretransfer editing has been estimated to account for 75% of the overall ATPase activity (*36*). On the basis of the model of Figure 7A, three rounds of processive pretransfer editing would occur before the tRNA dissociated and a new cycle of editing

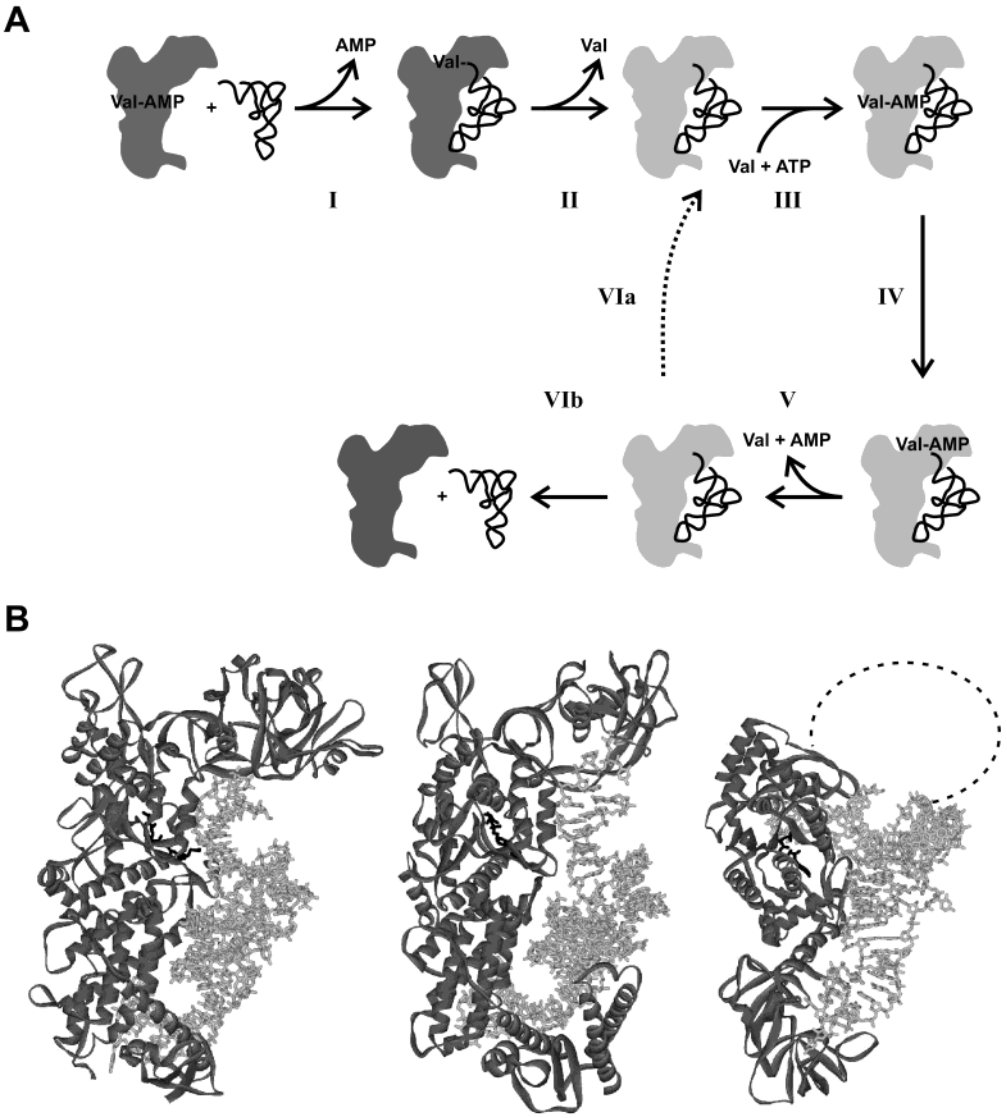

FIGURE 7: Post-transfer initiated pretransfer editing. (A) This editing model illustrates the dependence of all editing on deacylation of mischarged tRNA. Following the initial misactivation of valine, misacylation of $tRNA^{Ile}$ and translocation of the valyl group to the editing site is obligatory (**I**). Deacylation (**II**) without dissociation of the tRNA triggers the formation of an "editing active" complex (lighter gray shading). In this state the enzyme/tRNA complex is primed for pretransfer editing should another misactivation event (**III**) occur. Direct translocation of Val-AMP from the active site to the editing site (**IV**), followed by hydrolysis (**V**), and further misactivation (**VIa**) can occur processively while the tRNA remains bound. The "aminoacylation active" state (darker gray shading) is regenerated upon dissociation of the tRNA (**VIb**). (B) Left*:* Cocrystal structure of *T. thermophilus* IleRS bound to $tRNA^{Ile}$ (*11*). Middle: Cocrystal structure of *T. thermophilus* ValRS bound to $tRNA^{Val}$ (*14*). Right: Cocrystal structure of *E. coli* GlnRS bound to $tRNA^{Gln}$. The dotted line indicates the position of the editing domain (lacking in GlnRS) in the other two complexes.

was initiated. In contrast, for ValRS little processivity is seen, because most of the editing proceeds by the post-transfer route. The post-transfer initiated pretransfer model emphasizes the importance of deacylation to initiate formation of an editing active complex. While post-transfer editing is more critical within a cellular environment, this notion has largely been ignored. Mischarged tRNA has more potential for harm than does a misactivated adenylate. If a mischarged tRNA dissociates from an aminoacyl-tRNA synthetase it can directly participate in protein synthesis, generating an errant protein. A misactivated adenylate blocks the active site; yet, it is relatively harmless if it dissociates. For example, the half-life of Phe-AMP at 0 °C and neutral pH is only 12 min (*40*).

The editing sites of IleRS and ValRS may have first evolved the capability for post-transfer editing, as destruction of mischarged tRNA is vital to the fidelity of protein synthesis. As protein synthesis required higher and higher fidelity, a pretransfer mechanism appeared, but required an initial post-transfer event. Because the likelihood of two consecutive misactivation events is low, post-transfer editing must be the dominant editing mechanism in a cellular environment. In vitro, where only noncognate amino acid is presented to the synthetase, processive pretransfer editing becomes more prominent. Multiple editing pathways likely offered a selective advantage under a more diverse range of conditions throughout evolution. Under typical conditions post-transfer editing was vital, whereas during periods of exposure to high noncognate amino acid concentrations (perhaps due to the environment or poor metabolite regulation) the combination of pre- and post-transfer editing was essential. Higher overall editing may have also been more im-

portant if the synthetic active sites of ancestral synthetases were not yet as precise as current synthetases.

ACKNOWLEDGMENT

We thank Dr. Nancy Maizels and Dr. Alan Weiner for providing the plasmid encoding tRNA NTase. We are grateful to Dr. T. Hendrickson, Dr. T. Nomanbhoy, L. Nangle, Dr. A. Bishop, and Dr. M. Sprinzl for help in preparing materials and insightful discussion.

REFERENCES

1. Carter, C. W., Jr. (1993) Cognition, mechanism, and evolutionary relationships in aminoacyl-tRNA synthetases. *Annu. Rev. Biochem. 62*, 715−748.
2. Giege, R., Puglisi, J. D., and Florentz, C. (1993) tRNA structure and aminoacylation efficiency. *Prog. Nucleic Acid Res. Mol. Biol. 45*, 129−206.
3. Jakubowski, H., and Goldman, E. (1992) Editing of errors in selection of amino acids for protein synthesis. *Microbiol. Rev. 56*, 412−429.
4. Baldwin, A. N., and Berg, P. (1966) Transfer ribonucleic acid-induced hydrolysis of valyladenylate bound to isoleucyl ribonucleic acid synthetase. *J. Biol. Chem. 241*, 839−845.
5. Eldred, E. W., and Schimmel, P. R. (1972) Rapid deacylation by isoleucyl transfer ribonucleic acid synthetase of isoleucine-specific transfer ribonucleic acid aminoacylated with valine. *J. Biol. Chem. 247*, 2961−2964.
6. Fersht, A. R., and Kaethner, M. M. (1976) Enzyme hyperspecificity. Rejection of threonine by the valyl-tRNA synthetase by misacylation and hydrolytic editing. *Biochemistry 15*, 3342−3346.
7. Schmidt, E., and Schimmel, P. (1994) Mutational isolation of a sieve for editing in a transfer RNA synthetase. *Science 264*, 265−267.
8. Fersht, A. R., and Dingwall, C. (1979) Evidence for the double-sieve editing mechanism in protein synthesis. Steric exclusion of isoleucine by valyl-tRNA synthetases. *Biochemistry 18*, 2627−2631.
9. Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman and Company, New York.
10. Lin, L., Hale, S. P., and Schimmel, P. (1996) Aminoacylation error correction. *Nature 384*, 33−34.
11. Nureki, O., Vassylyev, D. G., Tateno, M., Shimada, A., Nakama, T., Fukai, S., Konno, M., Hendrickson, T. L., Schimmel, P., and Yokoyama, S. (1998) Enzyme structure with two catalytic sites for double-sieve selection of substrate. *Science 280*, 578−582.
12. Hendrickson, T. L., Nomanbhoy, T. K., and Schimmel, P. (2000) Errors from selective disruption of the editing center in a tRNA synthetase. *Biochemistry 39*, 8180−8186.
13. Silvian, L. F., Wang, J., and Steitz, T. A. (1999) Insights into editing from an Ile-tRNA synthetase structure with $tRNA^{Ile}$ and mupirocin. *Science 285*, 1074−1077.
14. Fukai, S., Nureki, O., Sekine, S., Shimada, A., Tao, J., Vassylyev, D. G., and Yokoyama, S. (2000) Structural basis for double-sieve discrimination of L-valine from L-isoleucine and L-threonine by the complex of $tRNA^{Val}$ and valyl-tRNA synthetase. *Cell 103*, 793−803.
15. Lincecum, T. L., Tukalo, M., Yaremchuk, A., Mursinna, R. S., Williams, A. M., Sproat, B. S., Van Den Eynde, W., Link, A., Van Calenbergh, S., Grotli, M., Martinis, S. A., and Cusack, S. (2003) Structural and Mechanistic Basis of Pre- and Posttransfer Editing by Leucyl-tRNA Synthetase. *Mol. Cell 11*, 951−963.
16. Hendrickson, T. L., Nomanbhoy, T. K., de Crécy-Lagard, V., Fukai, S., Nureki, O., Yokoyama, S., and Schimmel, P. (2002) Mutational Separation of Two Pathways for Editing by a Class I tRNA Synthetase. *Mol. Cell 9*, 353−362.
17. von der Haar, F., and Cramer, F. (1975) Isoleucyl-tRNA synthetase from baker's yeast: the 3′-hydroxyl group of the 3′-terminal ribose is essential for preventing misacylation of $tRNA^{Ile}$-C−C-A with misactivated valine. *FEBS Lett. 56*, 215−217.
18. von der Haar, F., and Cramer, F. (1976) Hydrolytic action of aminoacyl-tRNA synthetases from baker's yeast: "chemical proofreading" preventing acylation of $tRNA^{Ile}$ with misactivated valine. *Biochemistry 15*, 4131−4138.
19. Igloi, G. L., von der Haar, F., and Cramer, F. (1977) Hydrolytic action of aminoacyl-tRNA synthetases from baker's yeast. "Chemical proofreading" of Thr-$tRNA^{Val}$ by valyl-tRNA synthetase studied with modified $tRNA^{Val}$ and amino acid analogues. *Biochemistry 16*, 1696−1702.
20. Shiba, K., and Schimmel, P. (1992) Functional assembly of a randomly cleaved protein. *Proc. Natl. Acad. Sci. U.S.A. 89*, 1880−1884.
21. Fersht, A. R., Ashford, J. S., Bruton, C. J., Jakes, R., Koch, G. L., and Hartley, B. S. (1975) Active site titration and aminoacyl adenylate binding stoichiometry of aminoacyl-tRNA synthetases. *Biochemistry 14*, 1−4.
22. Nomanbhoy, T. K., and Schimmel, P. R. (2000) Misactivated amino acids translocate at similar rates across surface of a tRNA synthetase. *Proc. Natl. Acad. Sci. U.S.A. 97*, 5119−5122.
23. Döring, V., Mootz, H. D., Nangle, L. A., Hendrickson, T. L., de Crécy-Lagard, V., Schimmel, P., and Marliere, P. (2001) Enlarging the amino acid set of *Escherichia coli* by infiltration of the valine coding pathway. *Science 292*, 501−504.
24. Shi, P. Y., Weiner, A. M., and Maizels, N. (1998) A top-half tDNA minihelix is a good substrate for the eubacterial CCA-adding enzyme. *RNA 4*, 276−284.
25. Schmidt, E. (1996) Ph.D. Thesis, Massachusetts Institute of Technology, Cambridge, MA, p 160.
26. Glasfeld, E., Landro, J. A., and Schimmel, P. (1996) C-terminal zinc-containing peptide required for RNA recognition by a class I tRNA synthetase. *Biochemistry 35*, 4139−4145.
27. Francis, T. A., Ehrenfeld, G. M., Gregory, M. R., and Hecht, S. M. (1983) Transfer RNA pyrophosphorolysis with CTP(ATP): tRNA nucleotidyltransferase. A direct route to tRNAs modified at the 3′ terminus. *J. Biol. Chem. 258*, 4279−4284.
28. Shepard, A., Shiba, K., and Schimmel, P. (1992) RNA binding determinant in some class I tRNA synthetases identified by alignment-guided mutagenesis. *Proc. Natl. Acad. Sci. U.S.A. 89*, 9964−9968.
29. Nordin, B. E., and Schimmel, P. (2002) Plasticity of recognition of the 3′-end of mischarged tRNA by class I aminoacyl-tRNA synthetases. *J. Biol. Chem. 277*, 20510−20517.
30. Hale, S. P., and Schimmel, P. (1996) Protein synthesis editing by a DNA aptamer. *Proc. Natl. Acad. Sci. U.S.A. 93*, 2755−2758.
31. Cramer, F., Faulhammer, H., von der Haar, F., Sprinzl, M., and Sternbach, H. (1975) Aminoacyl-tRNA synthetases from baker's yeast: reacting site of enzymatic aminoacylation is not uniform for all tRNAs. *FEBS Lett. 56*, 212−214.
32. Freist, W., Sternbach, H., and Cramer, F. (1987) Isoleucyl-tRNA synthetase from baker's yeast and from *Escherichia coli* MRE 600. Discrimination of 20 amino acids in aminoacylation of tRNA-(Ile)-C−C-A(3′NH2). *Eur. J. Biochem. 169*, 33−39.
33. Taiji, M., Yokoyama, S., and Miyazawa, T. (1983) Transacylation rates of (aminoacyl)adenosine moiety at the 3′-terminus of aminoacyl transfer ribonucleic acid. *Biochemistry 22*, 3220−3225.
34. Fraser, T. H., and Rich, A. (1975) Amino acids are not all initially attached to the same position on transfer RNA molecules. *Proc. Natl. Acad. Sci. U.S.A. 72*, 3044−3048.
35. Freist, W., Pardowitz, I., and Cramer, F. (1985) Isoleucyl-tRNA synthetase from bakers' yeast: multistep proofreading in discrimination between isoleucine and valine with modulated accuracy, a scheme for molecular recognition by energy dissipation. *Biochemistry 24*, 7014−7023.
36. Fersht, A. R. (1977) Editing mechanisms in protein synthesis. Rejection of valine by the isoleucyl-tRNA synthetase. *Biochemistry 16*, 1025−1030.
37. Tardif, K. D., and Horowitz, J. (2002) Transfer RNA determinants for translational editing by *Escherichia coli* valyl-tRNA synthetase. *Nucleic Acids Res. 30*, 2538−2545.
38. Bishop, A. C., Nomanbhoy, T. K., and Schimmel, P. (2002) Blocking site-to-site translocation of a misactivated amino acid by mutation of a class I tRNA synthetase. *Proc. Natl. Acad. Sci. U.S.A. 99*, 585−590.
39. Rould, M. A., Perona, J. J., Söll, D., and Steitz, T. A. (1989) Structure of *E. coli* glutaminyl-tRNA synthetase complexed with tRNA(Gln) and ATP at 2.8 A resolution. *Science 246*, 1135−1142.
40. Illangasekare, M., and Yarus, M. (1997) Small-molecule-substrate interactions with a self-aminoacylating ribozyme. *J. Mol. Biol. 268*, 631−639.

BI035052Q