Editing by a tRNA Synthetase: DNA Aptamer-Induced Translocation and Hydrolysis of a Misactivated Amino Acid†

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ABSTRACT: Aminoacyl-tRNA synthetases establish the rules of the genetic code by aminoacylation reactions. Occasional activation of the wrong amino acid can lead to errors of protein synthesis. For isoleucyl-tRNA synthetase, these errors are reduced by tRNA-dependent hydrolytic editing reactions that occur at a site 25 Å from the active site. These reactions require that the misactivated amino acid be translocated from the active site to the center for editing. One mechanism describes translocation as requiring the mischarging of tRNA followed by a conformational change in the tRNA that moves the amino acid from one site to the other. Here a specific DNA aptamer is investigated. The aptamer can stimulate amino acid-specific editing but cannot be aminoacylated. Although the aptamer could in principle stimulate hydrolysis of a misactivated amino acid by an idiosyncratic mechanism, the aptamer is shown here to induce translocation and hydrolysis of misactivated aminoacyl adenylate at the same site as that seen with the tRNA cofactor. Thus, translocation to the site for editing does not require joining of the amino acid to the nucleic acid. Further experiments demonstrated that aptamer-induced editing is sensitive to aptamer sequence and that the aptamer is directed to a site other than the active site or tRNA binding site of the enzyme.

Aminoacyl-tRNA synthetases catalyze the transesterification of an amino acid onto the 3′ end of a tRNA. The accuracy of this process determines the fidelity of protein synthesis. To achieve the level of accuracy required for cell viability, a number of synthetases possess a second activity, i.e., the ability to correct misactivation of noncognate amino acids through hydrolytic editing reactions. For example, isoleucyl-tRNA synthetase (IleRS)[1] is a class I enzyme that has the problem of discriminating between isoleucine and valine. The *E. coli* enzyme misactivates valine in vitro at a frequency of about 0.5% compared to that of isoleucine (*1*). This error rate is reduced at least 20-fold by the editing activity of the enzyme, to give the observed level of misincorporation into protein of valine for isoleucine at a frequency of around 1 in 3000 (*2*).

The translational editing reaction catalyzed by IleRS is thought to occur via two possible pathways (Figure 1). The first of these is pretransfer editing, where the misactivated valine is directly hydrolyzed (*3*−*5*). The second route (posttransfer editing) involves the transfer of the amino acid to the 3′ end of the tRNA followed by the hydrolysis of the aminoacyl bond (*6*). Both activities require the cognate $tRNA^{Ile}$ (*7*).

The editing activity of IleRS is located within a 275 amino acid region—termed connective polypeptide 1 or CP1 (*8*)—inserted into the active site of the enzyme (*9*). The isolated CP1 domain was shown earlier to catalyze the deacylation of Val-$tRNA^{Ile}$ (*10*). Because the editing site is located some 25 Å away from the active site (*11*), the misactivated valine must be translocated from the active site to the editing center where it is hydrolyzed (*12*). Although a mechanism for the translocation of the misactivated valine following attachment to the tRNA has been proposed (*13*), this mechanism fails to explain how Val-AMP can be directly hydrolyzed.

A number of different class 1 synthetases—including methionyl-, leucyl-, valyl-, and isoleucyl-tRNA synthetases—have been shown to edit misactivated amino acids (*14*−*17*). In the case of MetRS, editing of homocysteinyl adenylate appears to occur by a pretransfer route within the active site of the enzyme (*18*). For ValRS, the majority of the reaction appears to proceed via a misacylated tRNA intermediate, namely, via the posttransfer route (*19*). For both ValRS and LeuRS (in addition to IleRS), the ability to remove the incorrect amino acid from the tRNA resides in the CP1 domain.

The ability of IleRS to hydrolyze misactivated valine is dependent upon the presence of its cognate tRNA (*7*). Furthermore, the tRNA must possess an intact 3′ end (*17*). This has made difficult the study and demonstration of the pretransfer pathway, because the presence of $tRNA^{Ile}$ makes possible the simultaneous occurrence of posttransfer editing. Although the pretransfer pathway was inferred from rapid

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[1] Abbreviations: IleRS, isoleucyl-tRNA synthetase; MetRS, methionyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; ValRS, valyl-tRNA synthetase; AMP, adenosine monophosphate; CP1, connective polypeptide 1; PP_i, pyrophosphate.

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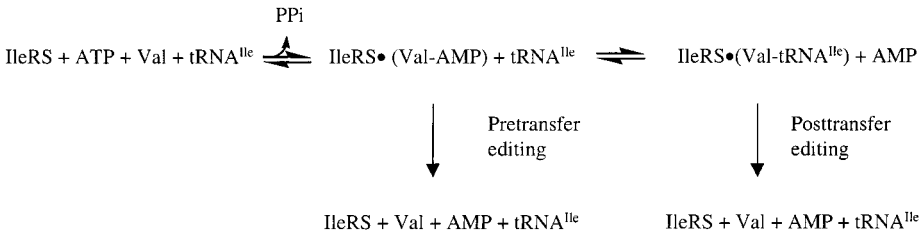

FIGURE 1: Editing reactions catalyzed by IleRS. Initially valine is misactivated to form Val-AMP. In the presence of $tRNA^{Ile}$, the valyl moiety can be either hydrolyzed directly (pretransfer editing) or transferred to the tRNA and subsequently hydrolyzed (posttransfer editing).

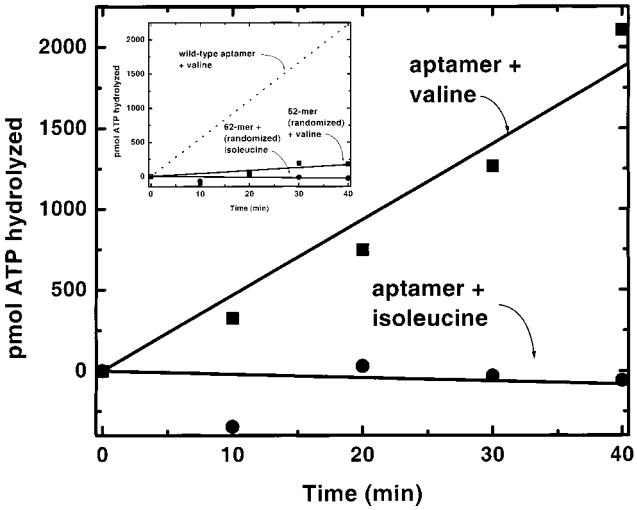

FIGURE 2: Aptamer-stimulated editing of either misactivated valine or isoleucine by IleRS at pH 7.8, 21 °C, as measured by the production of inorganic phosphate from ATP (after subtraction of the rate of ATP hydrolyzed following the addition of a 62-mer oligonucleotide of random sequence). The inset shows the effects of the randomized aptamer, with the background of 'no aptamer' subtracted.

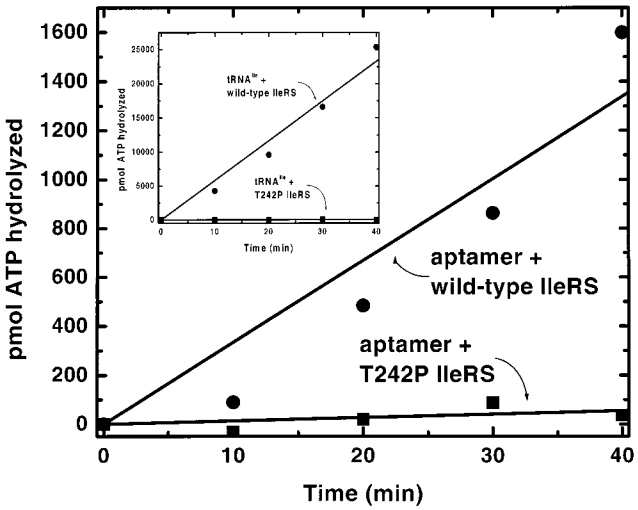

FIGURE 3: Editing by wild-type IleRS compared with IleRS containing a mutation in the editing site (T242P). The rate of editing is given by the consumption of ATP following subtraction of the rate in the presence of randomized 62-mer DNA and IleRS. The inset shows the tRNA-stimulated hydrolysis of ATP by wild-type and T242P IleRS.

kinetic studies (*3*), more recent experiments showed that a DNA aptamer could replace $tRNA^{Ile}$, even though the aptamer could not be aminoacylated (*5*, *20*). This observation provided further evidence for the existence of the pretransfer route. However, one possibility was that the aptamer was somehow stimulating hydrolysis of the Val-AMP within the active site and was thus involved in an idiosyncratic reaction not normally catalyzed by the enzyme. In this scenario, aptamer-induced editing could mimic in part the editing seen with MetRS (vide supra). Alternatively, the aptamer was stimulating hydrolysis at the center for editing in the CP1 domain. In this instance, it would be causing translocation of Val-AMP from the active site to the editing center. Thus, editing would occur at the canonical site, but translocation would be by a mechanism different from that previously proposed (*13*), where the aminoacyl group is bound to the tRNA. With this in mind, the present experiments were set out to demonstrate whether aptamer-induced editing occurred through a translocation event that brought the adenylate to the editing site in CP1, without ligation of the aminoacyl group to a nucleic acid.

MATERIALS AND METHODS

RNA Substrates. Natural *E. coli* $tRNA_1^{Ile}$(GAU) was produced from an overexpressing strain and isolated essentially as previously described. The concentrations of tRNA were determined by the plateau level of aminoacylation (*21*).

DNA Substrates. DNA oligonucleotides were obtained in desalted form from GIBCO−BRL (Rockville, MD) and were used without further purification. The wild-type sequence was based on that previously published (*20*). The purity of each oligonucleotide was confirmed by denaturing gel electrophoresis (*22*). The secondary structures of the DNA aptamers were predicted using RNAstructure 3.5 [D. H Mathews, M. E. Burkard, and D. H. Turner, University of Rochester, Rochester, NY (*23*)] where the DNA was folded as a single strand (*24*).

Protein Expression and Purification. 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 (*25*). The T242P mutant protein was expressed from *E. coli* MI1 cells transformed with pTLH24 (*26*). Proteins were isolated essentially as described (*21*), with purification on a size exclusion column (Superose 12, Pharmacia, Piscataway, NJ) being performed after ion-exchange chromatography. Enzyme concentrations were determined by active site titration (*27*).

ATPase Assays. Assays measuring the tRNA-dependent hydrolysis of misactivated valine (in the form of Val-AMP and Val-$tRNA^{Ile}$) were performed in 20 μL at room temperature (~20 °C) in 250 mM Tris-HCl (pH 7.8), 8 mM $MgCl_2$, 0.5 mM valine, 2.5 mM [γ-^{32}P]ATP (specific activity 2.5 μCi/μmol), 5 μg/mL inorganic pyrophosphatase (Roche Molecular Biochemicals, Indianapolis, IN), 1 μM IleRS, and either 12.5 μM $tRNA^{Ile}$ or 25 μM aptamer. The tRNA or

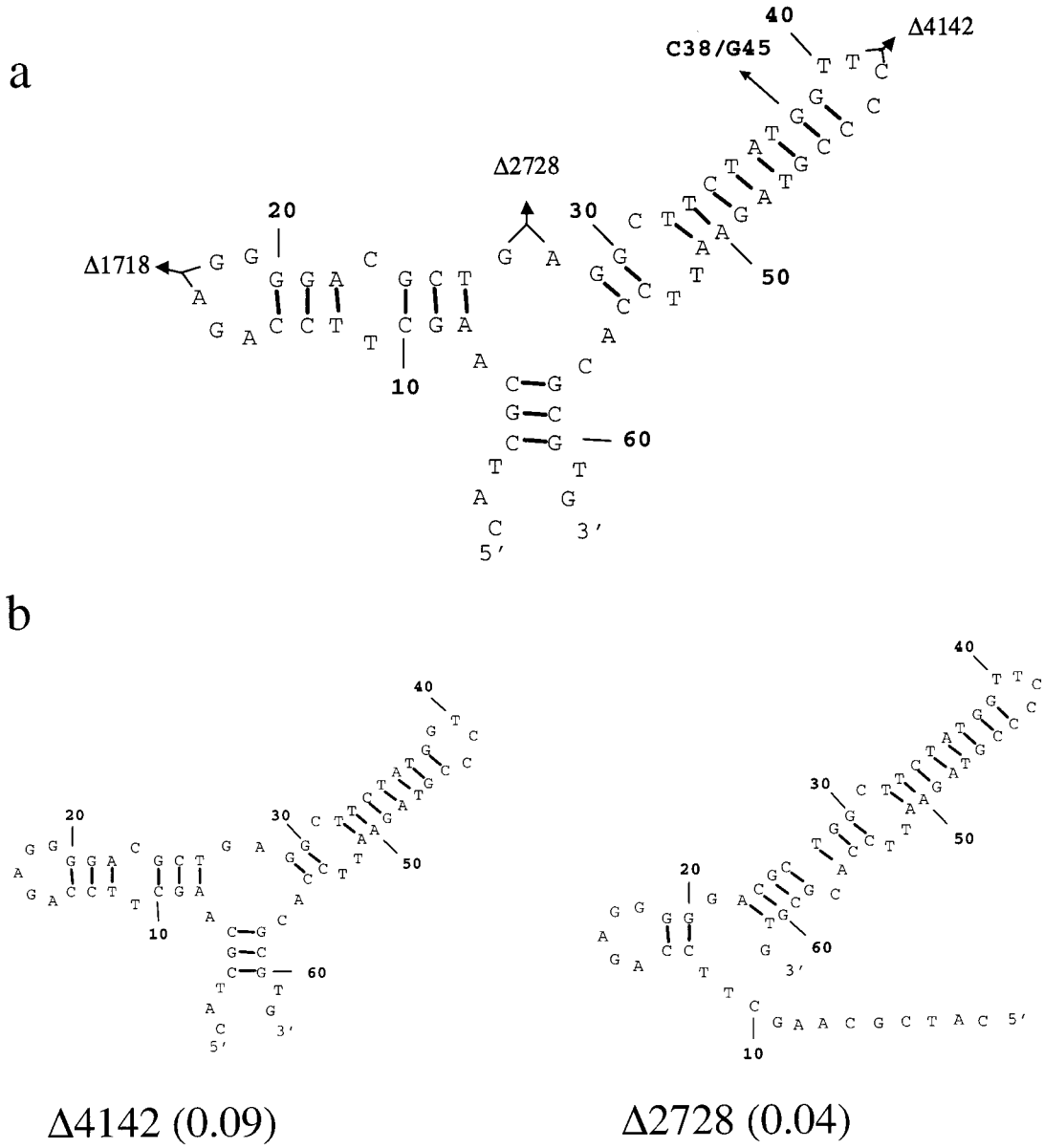

FIGURE 4: (a) Predicted secondary structure of the DNA aptamer showing the location of the mutations used in this study. The DNA was folded as a single strand using the program RNAstructure (*23*). (b) Predicted structures of the Δ2728 and Δ4142 mutant aptamers that are significantly reduced in editing are shown. The value in parentheses denotes the rate of editing relative to the wild-type aptamer, following subtraction of the rate obtained with the randomized 62-mer. The other mutations shown in (a) had a relatively small effect on the rate of editing.

DNA was heated to 85 °C in 60 mM Tris-HCl (pH 7.8), 2 mM $MgCl_2$, and cooled to room temperature before use. Aliquots (4.8 μL) 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 (*5*). For experiments to characterize the effects of the aptamer on $tRNA^{Ile}$-stimulated editing, IleRS was used at a concentration of 0.2 μM.

Aminoacylation Assays. Reactions were performed at room temperature in a 50 μL volume containing 250 mM Tris-HCl (pH 7.8), 8 mM $MgCl_2$, 20 μM [^{3}H]Ile (specific activity 800 μCi/μmol), and 10 nM IleRS, with either 12.5 μM $tRNA^{Ile}$ or 25 μM aptamer (or both). Before use, the nucleic acid was renatured as above. Aliquots (6 μL) of the reaction mixture were precipitated with trichloroacetic acid, and the level of aminoacylation of the tRNA was determined by scintillation counting (*21*).

RESULTS

Stimulation of Editing by a DNA Aptamer. The ability of the aptamer to stimulate editing was determined by measuring the rate of IleRS-catalyzed hydrolysis of ATP in the presence of valine (Figure 2). The presence of inorganic pyrophosphatase effectively ensures that the hydrolysis of ATP is irreversible. Under these conditions, the valine is continuously activated to give Val-AMP which is then hydrolyzed. However, addition of isoleucine in place of valine results in only one round of hydrolysis as Ile-AMP is formed but not broken down (Figure 2). In contrast to the addition of aptamer, the addition of a 62-mer DNA with a randomized sequence stimulates the rate of ATP hydrolysis by around 10% of the increase seen following the addition

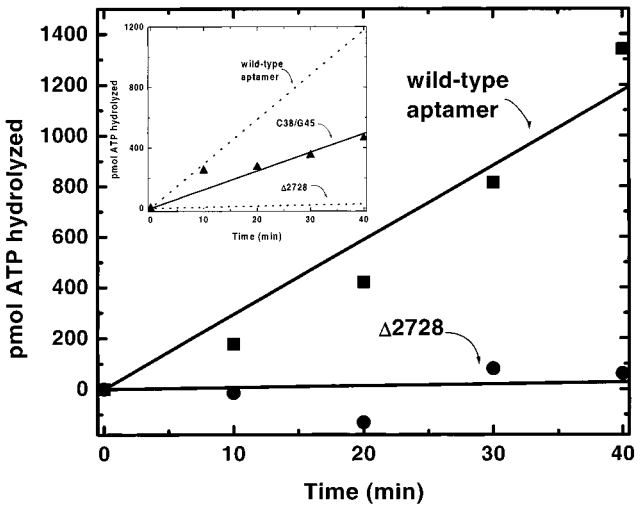

FIGURE 5: Stimulation of editing by wild-type aptamer compared with the Δ2728 mutation and with the C38/G45 transversion (inset) (after subtraction of the rate obtained following addition of the randomized 62-mer).

of wild-type aptamer. This increase showed little specificity for valine (Figure 2, inset).

Under the conditions of the assay, the stimulation of editing by the DNA aptamer is about 15% of that by the $tRNA^{Ile}$ (data not shown). This difference reflects in part that $tRNA^{Ile}$ can be misacylated with valine so that both pre- and posttransfer pathways occur. Because DNA cannot be aminoacylated, all aptamer-stimulated editing must be occurring via the pretransfer or another route (*5*).

A Mutation in the Editing Site in CP1 Abolishes Aptamer-Induced Editing. To determine whether editing stimulated by the aptamer occurred at the site within the CP1 domain, we took advantage of a mutant enzyme that was defective in the center for editing. Previous work showed that a T242P mutation in CP1 severely impaired the ability of the enzyme to catalyze the deacylation of Val-$tRNA^{Ile}$, thereby resulting in significant mischarging of $tRNA^{Ile}$ with valine (*26*). We wanted to determine what effect this mutation would have both on the overall editing reaction (as measured by the tRNA-stimulated hydrolysis of ATP) and on the pretransfer editing reaction (as stimulated by the DNA aptamer).

As found with wild-type $tRNA^{Ile}$, addition of aptamer to T242P IleRS also did not result in any stimulation of ATP hydrolysis (Figure 3). Thus, the editing activity stimulated by the aptamer depends on T242, just as it does for editing stimulated by $tRNA^{Ile}$. Because the misactivated valine cannot be attached to the DNA, this result means that a mechanism must exist for translocation of Val-AMP itself across the surface of the enzyme. The finding that the editing activity stimulated by the aptamer resides in the CP1 domain is consistent with earlier work that showed that the 3′ end of the aptamer could be cross-linked to amino acids within the CP1 insertion of IleRS (*20*). Because the 3′ end of $tRNA^{Ile}$ can also interact with the editing site in CP1, the results of cross-linking and mutagenesis (Figure 3) suggest that a direct interaction with CP1 is part of the mechanism of pretransfer editing.

Effects of Mutations in the Aptamer on Its Ability To Stimulate Editing. The aptamer used here was originally selected from a pool of approximately 10^{14} DNA oligonucleotides (*5*) (Figure 4a). The low number of hits achieved in

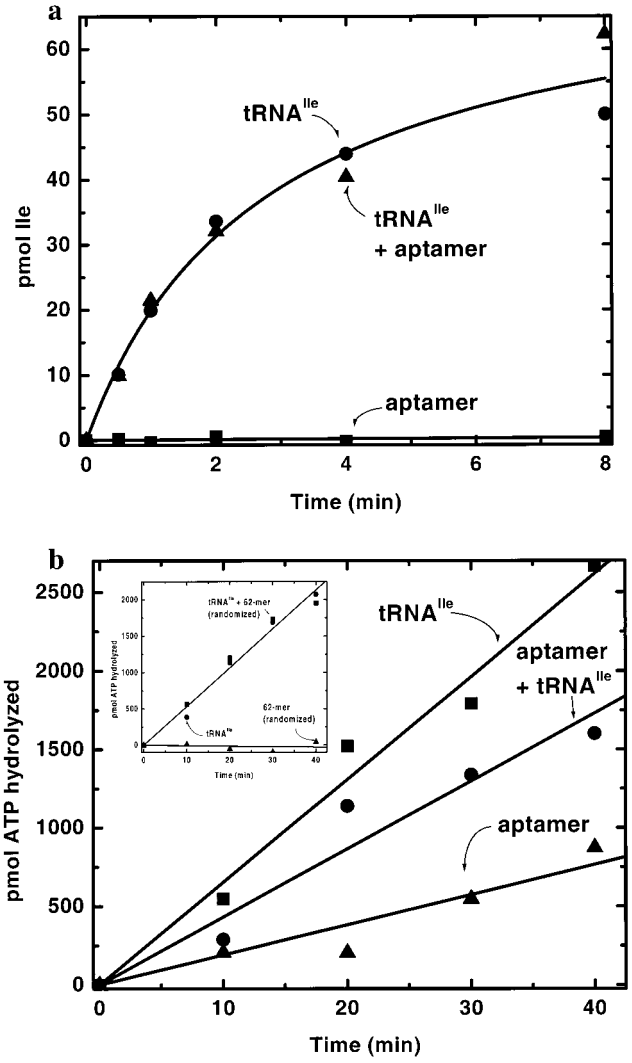

FIGURE 6: (a) Aminoacylation of $tRNA^{Ile}$, DNA aptamer, or $tRNA^{Ile}$ in the presence of aptamer at pH 7.8, 21 °C. (b) Editing of misactivated valine by IleRS stimulated by the addition of either $tRNA^{Ile}$, wild-type DNA aptamer, or both, at pH 7.8, 21 °C. The inset shows the effects of the randomized aptamer. The rate of ATP hydrolysis is given following subtraction of the rate in the presence of IleRS alone.

the selection suggested that a highly specific sequence/structure was required. To further investigate the sensitivity of the biological activity of the aptamer to its sequence, we examined whether changes in the predicted secondary structure of the DNA oligonucleotide would affect its ability to stimulate the editing reaction. This was done by making a number of mutations, some of which were predicted to cause a change in the secondary structure (Figure 4b) while others, although altering the sequence, maintained the predicted secondary structure.

The Δ2728 mutation was predicted by the nucleic acid folding program RNAstructure (*23*) to cause a large change in the conformation of the DNA (Figure 4b). This deletion resulted in a substantial drop in the aptamer-stimulated editing (Figure 5). In contrast, the C38/G45 transversion changed the sequence but maintained the predicted secondary structure. This substitution had a small effect on editing activity (Figure 5). A deletion in one of the predicted loops (Δ4142) also severely reduced the ability of the aptamer to stimulate editing and changed the predicted structure (see

Figure 4b). The effect of a mutation of the other loop (Δ1718) was more benign (60% reduction in activity), and this mutation did not substantially change the predicted secondary structure (data not shown). Thus, the editing of Val-AMP induced by the aptamer is highly sensitive to its sequence in a way that suggests that a specific structure is important for activity.

Effect of Aptamer on tRNA^{Ile}-Dependent Aminoacylation and Editing. As stated above, photo-cross-linking (with a 5-BrdU-substituted aptamer) showed that the 3′ end of the aptamer is directed to the CP1 domain (*20*). No cross-linking to the active site for aminoacylation was observed. Indeed, when aptamer was incubated with IleRS and tRNA^{Ile} in the aminoacylation reaction, no inhibition of aminoacylation was observed (Figure 6a). Because the aptamer does not stimulate editing as efficiently as tRNA^{Ile} [at least in part because the aptamer is not a substrate for aminoacylation (Figure 6a) so that a "mischarged" aminoacyl form is not produced], we imagined that the aptamer would partially reduce the editing that occurs in the presence of tRNA^{Ile} and valine. Under the same conditions where the aptamer does not inhibit aminoacylation, the overall ATPase activity associated with the editing of misactivated valine in the presence of tRNA^{Ile} was reduced significantly (Figure 6b). Addition of a randomized aptamer had no effect on editing (Figure 6b, inset). Thus, the aptamer is a highly specific probe for the editing site and can selectively affect the editing reaction without disruption of the catalytic center for aminoacylation.

DISCUSSION

The results reported here show clearly that a mechanism different than, or in addition to, that proposed previously must hold for the translocation pathway. Previous experiments demonstrated that translocation of misactivated valine from the active site to the CP1 domain is the rate-limiting step in tRNA-stimulated editing (*12*). Possibly, the lower rate of editing seen with the aptamer reflects in part a reduced rate of translocation of the misactivated adenylate across the surface of the enzyme.

Despite two crystal structures of IleRS, the mechanism by which Val-AMP is translocated some 25 Å remains obscure (*11, 13*). The stimulation of the editing reaction by tRNA^{Ile} is dependent upon the presence of specific nucleotides in the D-loop. These nucleotides are required for translocation but not for the hydrolytic step of the reaction (*7, 28*). Thus, they may be required to stimulate a conformational change in the protein, the tRNA, or both. Among the changes observed upon tRNA^{Ile} binding to IleRS are the following: a movement of the KMSKS loop away from the adenylate in the active site, the rotation of the CP1 domain by some 47° relative to the rest of the enzyme, and the formation of a "channel" between the active site and the editing site (*13*). As stated above, although the aptamer is less active than tRNA^{Ile}, part of the difference is due to the lack of a posttransfer pathway for the aptamer. In addition, the aptamer may only be capable of stimulating some, but not all, conformational changes, and this may account for its reduced ability to stimulate editing in comparison with tRNA^{Ile}.

The sequence specificity required for the aptamer to efficiently stimulate editing is reminiscent of the requirement for a particular sequence within the D-loop of tRNA^{Ile} to stimulate editing (*7, 28*). Intriguingly, the D-loop appears not to contact the protein directly (*11, 29*), raising the possibility that the correct D-loop sequence may be required to accurately position bases which are contacting the protein. In the absence of a structure for the aptamer, it is impossible to say whether the DNA is forming a structure that somehow mimics that of a portion of the tRNA including the D-loop, or whether the required contacts with the protein are being made by a totally different structure. Certainly, the predicted secondary structure (Figure 4a) has no obvious tRNA-like motif. Consistent with this lack of a tRNA-like motif is the ability of the aptamer to reduce tRNA-stimulated editing without altering the rate of aminoacylation of tRNA, suggesting that the aptamer is directed to a location distinct from the active site.

Although the aptamer may induce a conformational change that facilitates translocation, the proximity of the 3′ end of the aptamer to the editing center [as shown by cross-linking experiments (*20*)] makes tempting the speculation that the 3′ end of the nucleic acid may also be involved directly in the catalysis of hydrolysis of misactivated valine. Indeed, an intact 3′ end of the tRNA is required to stimulate editing, although the reason is not known (*17*). Further experiments with the aptamer are directed at this question.

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