# Errors from Selective Disruption of the Editing Center in a tRNA Synthetase[†]

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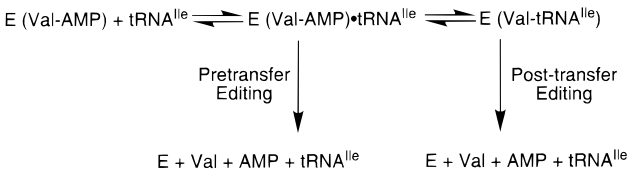
ABSTRACT: Some aminoacyl-tRNA synthetases have two catalytic centers that together achieve fine-structure discrimination of closely similar amino acids. The role of tRNA is to stimulate translocation of a misactivated amino acid from the active site to the editing site where the misactivated substrate is eliminated by hydrolysis. Using isoleucyl-tRNA synthetase as an example, we placed mutations in the catalytic center for editing at residues strongly conserved from bacteria to humans. A particular single substitution and one double substitution resulted in production of mischarged tRNA, by interfering specifically with the chemical step of hydrolytic editing. The substitutions affected neither amino acid activation nor aminoacylation, with the cognate amino acid. Thus, because of the demonstrated functional independence of the two catalytic sites, errors of aminoacylation can be generated by selective mutations in the center for editing.

In protein biosynthesis, aminoacyl-tRNA synthetases (AARSs)[1] catalyze the esterification of their cognate tRNAs with the appropriate amino acid, thereby connecting the trinucleotides of the genetic code to specific amino acids. To maintain the fidelity of this process, each AARS exhibits exquisite substrate specificity for its cognate amino acid and tRNA(s) substrates. The tRNA structure provides an array of recognition elements that facilitate selection by acting as either positive or negative identity elements (*1*). In contrast, discrimination between amino acids poses a significant problem in molecular recognition because the side chains are small and are often structurally similar. This problem in amino acid selection is best exemplified with the amino acids isoleucine and valine, which are both $\beta$-branched and whose side chains differ by only one methylene group. Based on the energy contributions of one methylene group, valine misincorporation at isoleucine codons was predicted to be as high as 20% in protein biosynthesis (*2*). However, biochemical analyses revealed that the actual error rate is less than 1 in 3000 (*3*). In this and other extreme cases, high levels of accuracy are ensured only because the corresponding AARSs have evolved tRNA-dependent editing reactions which hydrolytically cleave noncognate intermediates before misacylated tRNA is released from the enzyme (*4*).

Isoleucyl-tRNA synthetase (IleRS) catalyzes the specific aminoacylation of $tRNA^{Ile}$ with isoleucine (Ile) to generate Ile-$tRNA^{Ile}$, even in the presence of the structurally similar but noncognate amino acid valine (*4*). IleRS, like all AARSs, uses a two-reaction pathway to generate aminoacylated tRNA. First, Ile is condensed with one molecule of ATP to generate an isoleucyl adenylate (Ile-AMP, amino acid activation step); in a second reaction, the activated Ile is transferred from the adenylate to the 3′-end of $tRNA^{Ile}$ (aminoacylation step). To efficiently bind and activate Ile, the active site of IleRS inadvertently accommodates and processes smaller amino acids such as valine. In fact, valyl adenylate (Val-AMP) is formed with a $k_{cat}/K_m$ of activation that is only 180-fold lower than that for Ile (*5*). Some of the activated valine is also transiently transferred to $tRNA^{Ile}$ (*6*). According to current thinking, two tRNA-dependent editing reactions guarantee that both Val-$tRNA^{Ile}$ and Val-AMP are hydrolyzed before valine can be misincorporated into nascent proteins. These tRNA-dependent editing reactions are shown in Scheme 1.

Scheme 1




Pretransfer editing is the hydrolysis of Val-AMP; this reaction is $tRNA^{Ile}$-dependent, but proceeds without the formation of a Val-$tRNA^{Ile}$ intermediate (*7*, *8*). Posttransfer editing constitutes the deacylation of Val-$tRNA^{Ile}$ after the valine has been transferred to the 3′-end of the tRNA (*9*). Both reactions generate valine and AMP as the reaction products. Thus, the overall editing reaction can be observed as a continuous hydrolysis of ATP.

Biochemical and genetic experiments demonstrated that the editing site of IleRS is functionally distinct from the

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[1] Abbreviations: AARS, aminoacyl-tRNA synthetase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CP1, connective polypeptide 1; dATP[†], *N*-methylanthraniloyl dATP; IleRS, isoleucyl-tRNA synthetase; $PP_i$, inorganic pyrophosphate; TCA, trichloroacetic acid; ValRS, valyl-tRNA synthetase.

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synthetic active site. The synthetic active site of IleRS was first identified by homology modeling studies with the known structures of class I tRNA synthetases (*10*). It consists of a classic Rossman fold, an ATP binding domain that includes the canonical 11 amino acid signature sequence ending in HIGH and the KMSKS pentapeptide that are conserved in the 10 class I AARSs (*10*−*13*). A G56A mutation within this Rossman fold generates a variant of IleRS that has the same $k_{cat}/K_m$ for valine as for isoleucine (in the formation of the synthesis of the aminoacyl adenylate) (*5*). This enzyme does not generate Val-tRNA$^{Ile}$ because the deacylation of this mischarged tRNA is unaffected by the G56A mutation. Cross-linking studies localized the editing site to a large insertion (CP1) that bisects the synthetic active site between the third and fourth $\beta$ strands of the Rossman fold (*14*, *15*). IleRS variants containing point mutations near the cross-linking site (including H403Q and Y403F) altered the ability of IleRS to discriminate between Ile and Val in the deacylation of Val-tRNA$^{Ile}$ versus Ile-tRNA$^{Ile}$ (*14*). Subsequently, the CP1 domain alone was cloned out of IleRS and ValRS, respectively, and shown to encode the editing activity (*16*).

Two crystal structures of IleRS have recently been reported: the first is *T. thermophilus* IleRS (*17*), and the second is *S. aureus* IleRS in a complex with tRNA$^{Ile}$ (*18*). In both structures, the synthetic site and the editing site are separated by more than 25 Å, indicating that the substrates for editing must be translocated from the active site into the editing site. As originally proposed, these two distinct catalytic centers comprise a "double sieve" mechanism of substrate selection (*19*). The catalytic site of the enzyme, or coarse sieve, discriminates against all amino acids that are larger than isoleucine but, by necessity, accidentally activates some smaller amino acids. The second "fine" sieve is the editing site which precisely eliminates via hydrolysis both Val-AMP and Val-tRNA$^{Ile}$. The two sites are tied together by tRNA$^{Ile}$-induced translocation of substrates for editing from the active site to the editing site.

As part of the characterization of the crystal structure of IleRS from *T. thermophilus*, one molecule of Ile was demonstrated to bind in the synthetic site; in contrast, two molecules of valine could bind, one at the active site and one at the editing site in CP1 (*17*). An examination of the CP1-bound valine offered the first clear picture of the editing site of IleRS. The hydrophobic side chain of valine was nestled between a conserved tryptophan and a tyrosine; the tyrosine corresponds to Tyr403 that in *E. coli* IleRS was shown by mutational analysis to be involved in discrimination of Val versus Ile in the posttransfer deacylation reaction (*14*). The valine carboxylate was adjacent to a threonine-rich, highly conserved region of IleRS. Alanine substitutions of two residues in this threonine-rich peptide (T242A and N250A) generated variants of IleRS that are diminished in their editing activity (*17*, *20*).

In this work, we report results of a further evaluation of the editing site of IleRS. In particular, we addressed the question of whether mutations could be introduced into the editing site that substantially enhanced the ability to produce Val-tRNA$^{Ile}$. To be successful, we recognized that the editing site would need to be functionally independent of the active site, so that activation of valine would freely occur despite alterations to the editing domain in CP1. Earlier experiments with mutations in CP1 had suggested the possibility of such independence, but all of the previous mutants (e.g., H401Q and Y403F; see Figure 1) were still robust in the deacylation of Val-tRNA$^{Ile}$ (*5*, *14*). For these experiments, we did further manipulations of T242 and N250 in CP1, because of their close proximity to bound valine in the crystal structure of *T. thermophilus* IleRS and because earlier work suggested that editing was sensitive to the nature of the residue at this position (*17*).

## MATERIALS AND METHODS

*Plasmid Construction.* Construction of the plasmid containing the T242A mutation in *ileS* (using a three-primer-PCR method) has been previously reported (*21*). This same method was used to generate a plasmid (pTLH5) encoding for the N250A mutation in IleRS. In this case, the mutagenic primer contained the desired AAC to GCC mutation. This method of PCR amplification was also used to construct a new plasmid (pTLH23) in which the *Nco*I−*Bgl*II fragment of IleRS-encoding pKS21 (*22*, *23*) was replaced with a PCR-amplified fragment containing two new and unique restriction sites (*Spe*I and *Cla*I). These sites flank the region of *ileS* that encodes residues 239−252 and were designed so that the *Spe*I/*Cla*I fragment can be replaced by synthetic oligonucleotides. The T242P (encoded by plasmid pTLH24) and T242A/N250A (plasmid pTLH25) mutations were introduced into *ileS* by the ligation of oligonucleotides with the appropriate mutations (T242P-ACC to CCC; T242A/N250A to ACC/GCG and AAC/GCC) into the *Spe*I and *Cla*I sites of pTLH23. Each plasmid construction was verified by DNA sequence analysis.

*Protein Expression and Purification.* Wild-type monomeric IleRS was overexpressed in the *E. coli* K-12 strain MV1184 from the multi-copy plasmid pKS21, which contains *ileS* under the control of the *lac* promoter (*22*, *23*). Mutant proteins T242A and N250A were overexpressed and purified as previously described for the T242A mutant protein (*21*). Plasmids pTLH24 and pTLH25 were independently transformed and overexpressed in the *E. coli* strain MI1 (*24*, *25*). This strain contains a mutant *ileS* allele which introduces an F570S mutation into the synthetic active site of the chromosome-encoded IleRS (*5*). This mutation introduces an isoleucine-dependent growth phenotype, due to an elevated $K_m$ (>5 mM) for Ile. Both mutant proteins (T242P and T242A/N250A) were overexpressed and purified at levels approximately 10−20-fold higher than the chromosomally encoded F570S enzyme. Thus, the plasmid-expressed mutant proteins are expected to be contaminated with low levels (<10%) of the encoded F570S mutant IleRS. The high $K_m$ for Ile exhibited by this contaminant enzyme renders it silent in both amino acid activation and aminoacylation, under standard conditions (see below). The contaminating enzyme has wild-type activity for posttransfer editing (deacylation of Val-tRNA$^{Ile}$), but it does not catalyze the formation of Val-tRNA$^{Ile}$ (*5*). Therefore, the observed misaminoacylation of tRNA$^{Ile}$ (see below) can only be due to the purified proteins bearing mutations in CP1.

*Aminoacylation and Amino Acid Activation Assays.* Prior to kinetic assays, the concentration of the active site of each protein solution was determined in triplicate by titration with isoleucine and radiolabeled ATP (data not shown)(*26*). Wild-type IleRS and the T242A, N250A, T242P, and T242A/

N250A mutant enzymes were evaluated for their ability to aminoacylate and misaminoacylate $tRNA^{Ile}$ with Val and Ile, respectively. Aminoacylation assays with Ile were performed at 37 °C as previously described with $tRNA^{Ile}$ (4 $\mu$M), [$^3$H]-Ile (20 $\mu$M, 800 dpm/pmol, $K_m \sim 5$ $\mu$M), and AARS (5 nM wild-type IleRS or the appropriate mutant enzyme) (*27*). The ability of each enzyme to misaminoacylate $tRNA^{Ile}$ with valine was evaluated according to the same protocol with $tRNA^{Ile}$ (4 $\mu$M), [$^3$H]Val (2 $\mu$M, $K_m \sim 500$ $\mu$M, 10 000 dpm/pmol), and AARS (500 nM wild-type or mutant enzyme). (In these assays, a subsaturating concentration of valine was used to ensure a high specific activity in order to facilitate detection.)

Amino acid activation was evaluated at ambient temperature by monitoring the isoleucine-dependent ATP−pyrophosphate ($PP_i$) exchange reaction (*28*). Each reaction mixture contained Tris-HCl (100 mM, pH 7.5), $\beta$-mercaptoethanol (5 mM), $MgCl_2$ (5 mM), ATP (2 mM), $NaPP_i$ (2 mM, 2000 cpm/nmol), bovine serum albumin (100 $\mu$g/mL), Ile (10 mM), and wild-type IleRS or the appropriate mutant enzyme (100 nM). Aliquots were removed at various times and quenched into a solution that contained $NaPP_i$ (33 mM), 7% perchloric acid, and 8.3% (w/v) activated charcoal. The charcoal was filtered through a 0.45 $\mu$m centrifugal filter (Z-Spin Plus, Gelman Sciences) and washed twice with a solution containing 10 mM $NaPP_i$ and 0.5% perchloric acid. Charcoal-absorbed [$^{32}$P]ATP was quantitated by scintillation counting of the filter and activated charcoal. All assays were done in triplicate.

*Deacylation of Val-tRNA$^{Ile}$.* Val($^3$H)-$tRNA^{Ile}$ was prepared as described (*5*, *29*). Deacylation assays were performed at 25 °C in Tris-HCl (150 mM, pH 7.5), bovine serum albumin (100 $\mu$g/mL), $MgCl_2$ (10 mM), Val($^3$H)-$tRNA^{Ile}$ (3 $\mu$M, 1333 dpm/pmol), wild-type IleRS, or T242P or T242A/N250A mutant IleRS (1 $\mu$M). Aliquots were removed at specific time points and quenched onto filter pads (Whatman, 3 mm) that had been equilibrated with 5% trichloroacetic acid (TCA). Pads were washed 3 times with 5% TCA and once with 95% ethanol. The radioactive precipitate was quantitated by scintillation counting.

*Resonance Energy Transfer Assay for Translocation*. Fluorescence measurements were made using a Perkin-Elmer LS 50 Luminescence Spectrometer (Perkin-Elmer Ltd., Beaconsfield, Buckinghamshire, England). Samples were stirred continuously at room temperature (~20 °C) in 20 mM HEPES (pH 7.5), 50 mM NaCl, and 1 mM $MgCl_2$. Translocation of misactivated valine was monitored using the fluorescent ATP analogue *N*-methylanthraniloyl dATP (dATP$^\dagger$) as previously described (*21*). Either wild-type, T242A, T242A/N250A, or T242P IleRS (800 nM) was incubated for 4 min with dATP$^\dagger$(30 $\mu$M), valine (0.25 mM), ATP (750 nM), and pyrophosphatase (4 nM) (to ensure that all IleRS was complexed to Val-AMP). The energy-transfer-dependent fluorescence of dATP$^\dagger$ was observed (excitation of tryptophans in IleRS at 295 nm, emission of dATP$^\dagger$ at 440 nm), and $tRNA^{Ile}$ (120 nM) was added to stimulate translocation of Val-AMP. The fluorescence traces were normalized to correct for the quenching of the energy-transfer-dependent fluorescence of dATP$^\dagger$ caused by the inner-filter effect from $tRNA^{Ile}$, and the binding of $tRNA^{Ile}$ to IleRS. Despite the high background of dATP$^\dagger$ fluorescence, the signal-to-noise ratio was generally 20:1(*21*).

## RESULTS

*Val-tRNA$^{Ile}$ Formation by Wild-Type and Mutant IleRS.* In the crystal structure of IleRS complexed with valine bound to the editing site in CP1, the valine is situated in a hydrophobic binding pocket adjacent to a highly conserved threonine-rich segment (Figure 1, top) (*17*). Mutational analyses of this region revealed four residues along the wall of this pocket (T242, N250, H401, and Y403) which bear chemical functionalities that are important for wild-type editing (Figure 1, center) (*5*, *17*). In all published sequences for IleRS, these four residues and significant portions of the surrounding sequences are rigorously conserved from bacteria to humans (Figure 1, bottom).

To obtain mutant proteins that were capable of mischarging $tRNA^{Ile}$, we focused on T242 and N250 because preliminary work suggested that single alanine replacements at either position could result in a weak mischarging phenotype (*20*). After more thoroughly characterizing these mutant enzymes, our strategy was to make more dramatic substitutions to see if the mischarging phenotype could be enhanced. Each mutant enzyme (T242A and N250A) was evaluated for the ability to catalyze the formation of Val-$tRNA^{Ile}$. Although the Asn250 side chain is distal to the valine binding pocket (Figure 1, top), the N250A mutation generated an enzyme that weakly, but detectably, misacylated $tRNA^{Ile}$ (Figure 2). In contrast, T242A IleRS generated more Val-$tRNA^{Ile}$ than did the N250A mutant enzyme. The difference in behavior between the two mutant proteins is possibly because of the closer proximity of T242 to the valine pocket.

Based on the results with these mutations, second generation mutants were introduced into the same positions with the idea of more drastically disrupting the editing center of IleRS. Two new mutant enzymes were constructed and overexpressed. These contained either a T242P point mutation or a T242A/N250A double mutation. Both of these IleRS variants catalyzed the formation of Val-$tRNA^{Ile}$ to levels more than an order of magnitude higher than did the T242A and N250A mutant enzymes (Figure 2). Interestingly, the level of mischarging which resulted from the T242A/N250A double mutation was synergistically enhanced compared to that predicted from the sum of the two effects of the individual point mutations. The T242P and T242A/N250A substitutions are the first examples of point mutations in the editing center of an AARS that induce significant errors of aminoacylation.

*Point Mutations in the Editing Center of IleRS Are Deficient in Deacylation of Val-tRNA$^{Ile}$.* The two new mutant enzymes, T242P and T242A/N250A, were tested for their ability to catalyze the deacylation of Val-$tRNA^{Ile}$ (*9*). To detect low rates of deacylation, the assays were performed at high levels of each mutant enzyme. Both the T242P and the T242A/N250A mutant enzymes were defective in deacylation of Val-$tRNA^{Ile}$, even at high concentrations of each enzyme (Figure 3). [An identical slow rate of deacylation was observed for both enzymes. We believe this residual level of deacylation arises from trace amounts of the F570S mutant IleRS that is present in the enzyme preparation (see Materials and Methods).]

*Synthetic Active Site Is Unaffected by Point Mutations in the Editing Center.* The introduction of specific point

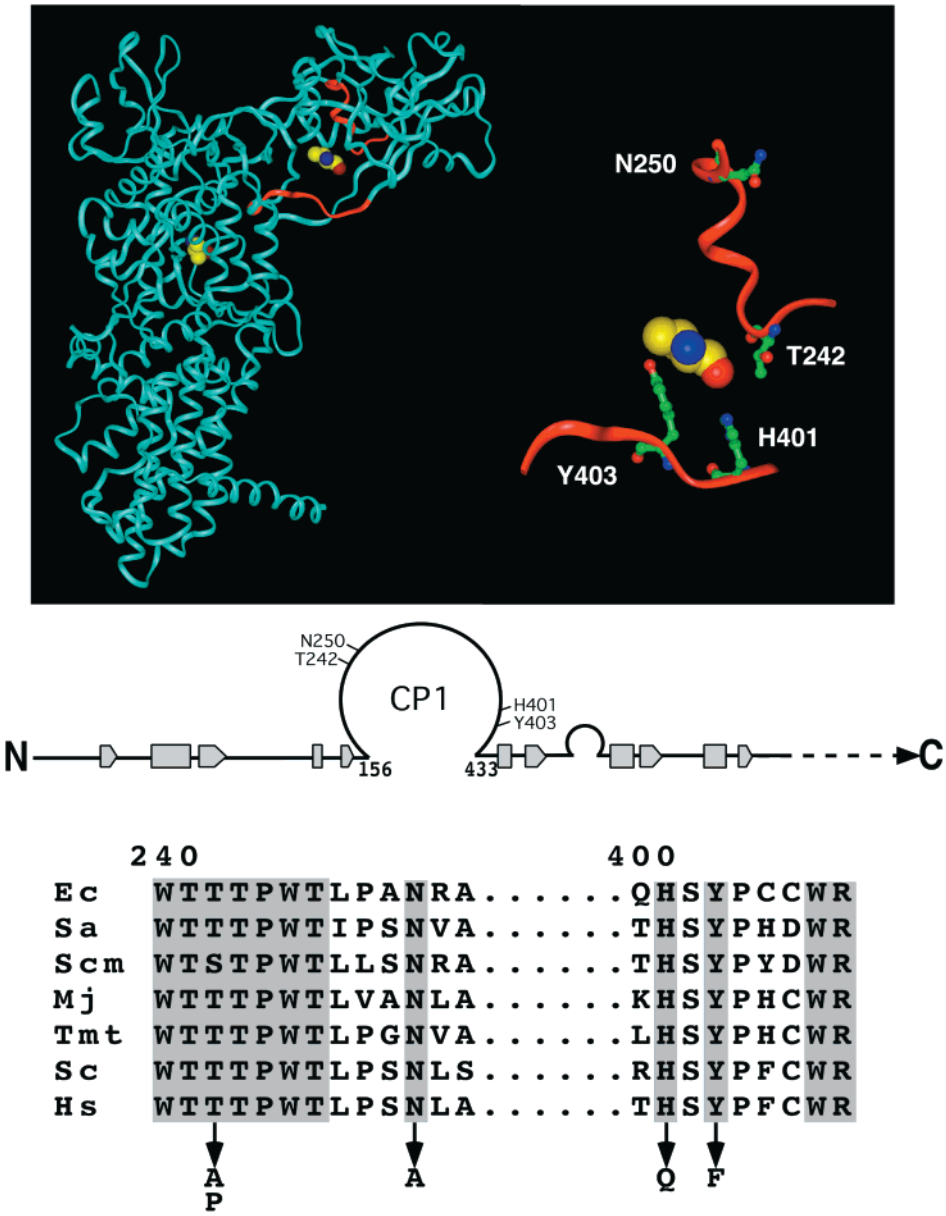


FIGURE 1: Editing site of IleRS is distinct from the synthetic active site. (Top) The crystal structure of IleRS from *T. thermophilus* complexed with two valines (yellow), one in the active site and one in the editing site. The editing site is highlighted in red (see alignment below). The side chains of residues which have been implicated in editing are shown in the expansion on the right (residue numbering system is based on the sequence of *E. coli* IleRS). (Middle) The IleRS editing site (CP1) intersects the alternating $\beta$-strands (pentagons) and α-helices (rectangles) of the nucleotide binding fold domain. (Bottom) Alignment of conserved residues in the editing site of IleRS. Seven representative sequences are shown. Residues highlighted in gray are rigorously conserved among all published sequences including bacteria, archaea, and eukaryotes (>30 sequences).

mutations into the editing center of IleRS results in enzymes that inaccurately misacylated $tRNA^{Ile}$ with valine. To establish that this observation is solely due to errors in editing, and not also due to indirect effects of the mutations on the catalytic active site, each mutant enzyme was evaluated for amino acid activation and for aminoacylation of $tRNA^{Ile}$ (with isoleucine). Amino acid activation was investigated by monitoring the isoleucine-dependent ATP−$PP_i$ exchange reaction (*28*). This activity was unaffected by the mutations in the editing center (Figure 4, inset). Each mutant enzyme also catalyzed the formation of Ile-$tRNA^{Ile}$ with rates indistinguishable from the rate observed with wild-type IleRS (Figure 4). Thus, the active site for adenylate synthesis and aminoacylation was unaffected by point mutations in the editing site. This result mirrors previous data that conversely showed that mutations in the active site for aminoacylation did not affect the ability of IleRS to catalyze the deacylation of mischarged tRNA (*5*). Thus, the present study, when combined with earlier work, shows that each site can be mutationally isolated from the other.

*tRNA-Dependent Translocation of Misactivated Valine by Mutant Enzymes.* We recently developed a fluorescence energy transfer assay that directly monitors the translocation of misactivated valine during editing (*21*). The fluorescent nucleotide *N*-methylanthraniloyl dATP (dATP†) binds the active site with a $K_d$ of approximately 2 $\mu$M. This binding can be observed by energy transfer between the tryptophan residues of IleRS and dATP†. Excitation of the IleRS·dATP† complex at 295 nm leads to energy-transfer-dependent emission at 440 nm. The aminoacyl adenylates (Ile-AMP

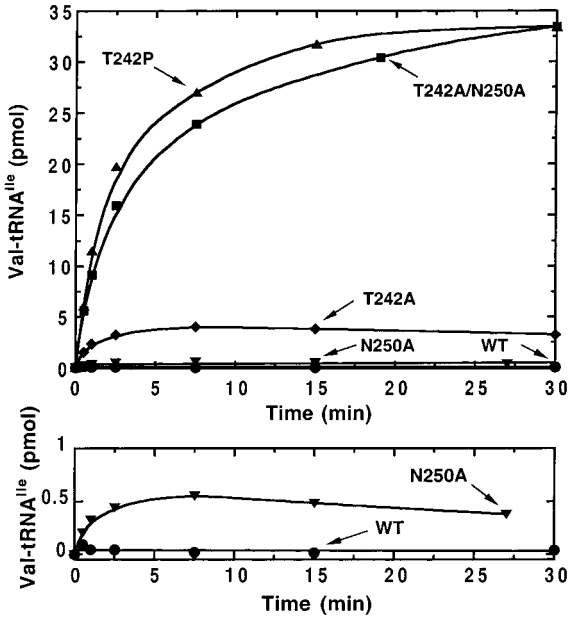


FIGURE 2: Misaminoacylation of tRNA$^{Ile}$ with valine at pH 7.5 and 37 °C. Wild-type and mutant IleRSs were assayed for their ability to misacylate tRNA$^{Ile}$ with valine. Top Panel: Enzymes containing the three single mutations (T242A, N250A, T242P) and the double mutation (T242A/N250A) misacylate tRNA$^{Ile}$ with valine. Wild-type IleRS does not catalyze this misacylation reaction. Bottom Panel: Expansion of data for IleRS with the N250A point mutation. This mutant enzyme misacylates tRNA$^{Ile}$ at low but detectable levels.

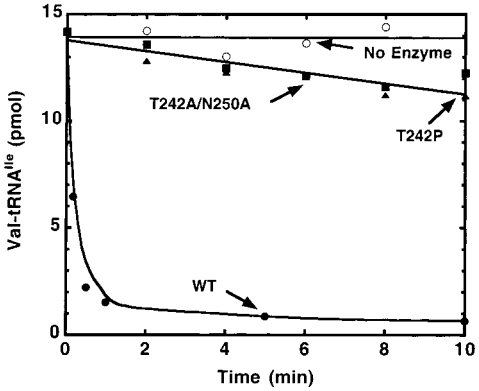


FIGURE 3: Posttransfer editing by wild-type IleRS and the T242P and T242A/N250A mutant enzymes at pH 7.5 and ambient temperature. Each enzyme was assayed for posttransfer editing by monitoring the enzymatically catalyzed deacylation of Val-tRNA$^{Ile}$. Both T242P and T242A/N250A IleRS enzymes are severely defective in editing.

and Val-AMP) have a much higher affinity for the active site ($K_d$ < 10 nM) than does dATP$^{\dagger}$, and are therefore able to competitively displace dATP$^{\dagger}$. Thus, the energy-transfer-dependent emission of dATP$^{\dagger}$ measures the fractional occupancy of the active site with dATP$^{\dagger}$ versus with aminoacyl adenylate.

After dATP$^{\dagger}$ is displaced by Val-AMP, the addition of tRNA$^{Ile}$ leads to translocation and editing. The misactivated valine (either as Val-AMP or as Val-tRNA$^{Ile}$) is translocated to the editing site where it is subsequently hydrolyzed (*21*). The translocation of misactivated valine results in the emptying of the active site, thus allowing the rebinding of dATP$^{\dagger}$. This, in turn, results in energy-transfer-dependent fluorescence at 440 nm. The rate of this fluorescence increase

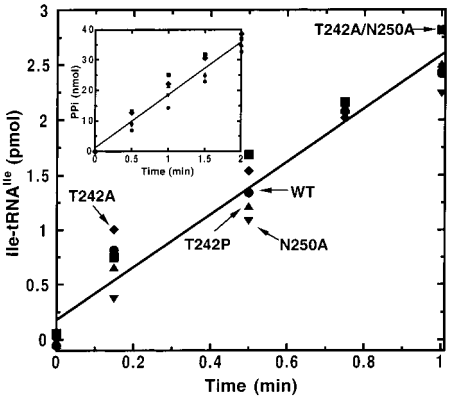


FIGURE 4: Aminoacylation and amino acid activation by wild-type and mutant IleRS enzymes at pH 7.5 and either 37 °C (aminoacylation) or ambient temperature (activation). Each enzyme was evaluated for its ability to aminoacylate tRNA$^{Ile}$ with Ile, and compared with wild-type IleRS. (Inset) Activation of Ile to form isoleucyl adenylate was monitored by ATP−PP$_i$ exchange. Point mutants in the IleRS editing site do not affect the kinetics of either reaction.

directly measures the kinetics of translocation of misactivated valine.

[Because the bimolecular step of binding and dissociation of tRNA$^{Ile}$ is kinetically coupled to the unimolecular translocation, the apparent rate of translocation can be reduced by using a low concentration of tRNA$^{Ile}$ (below the $K_D$ of 150 nM). In this way, we were able to measure the rate of translocation for mutant and wild-type enzymes in the time range of seconds. In addition, the concentration of ATP was chosen to ensure that less than one round of adenylate formation and translocation would occur. This situation enables us to avoid deconvoluting data representing multiple translocation events.]

We had previously used this assay to establish that, although the T242A mutant enzyme is defective in the hydrolysis of misactivated valine, it translocates misactivated valine from the active site to the editing site at a rate similar to that of the wild-type enzyme [(*21*) and data not shown]. Here, we used the fluorescence assay to examine the translocation of misactivated valine by the T242P and T242A/N250A mutant enzymes (Figure 5). Relative to wild-type IleRS, both mutant enzymes showed a 2-fold decrease in the rate of translocation of misactivated valine. Thus, these particular point mutants have only slightly impaired the rate of translocation of misactivated valine. This result further establishes that the mutations at T242 and N250 predominantly affect the chemical step of hydrolysis of misactivated valine.

## DISCUSSION

We presented here evidence that specific residues within the CP1 insertion of IleRS are essential for the accurate generation of Ile-tRNA$^{Ile}$; mutations at either T242 or N250 created mutant IleRS variants that misacylated tRNA$^{Ile}$ with Val. These observations suggest that, in the wild-type enzyme, T242 and N250 play important roles in the hydrolytic editing activity of the enzyme. It is unlikely that either residue is directly involved in catalysis, because neither the T242A nor the N250A mutant enzyme is completely defective in editing. Thus, it remains of interest to identify

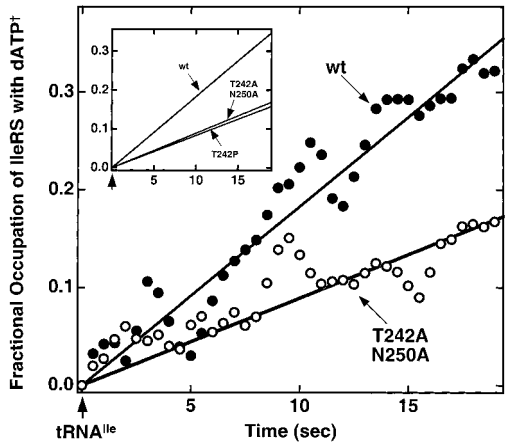


FIGURE 5: tRNA-dependent translocation of misactivated valine by wild-type and mutant IleRS enzymes as monitored by resonance energy transfer (at ambient temperature and pH 7.5). Val-AMP was first enzymatically generated in the synthetic active site. Translocation from the synthetic active site to the editing site is induced by the edition of $tRNA^{Ile}$ (arrow). The clearance of misactivated valine from the synthetic site allows the rebinding of the fluorescent ATP analogue dATP†, resulting in an increase in energy-transfer-dependent emission at 440 nm (*21*). Under single-turnover conditions (see description under Materials and Methods), this signal is directly related to the rate of translocation. A subsaturating amount of $tRNA^{Ile}$ was used in order to reduce the observed overall rate of translocation so that each mutant enzyme could be examined in close detail.

the precise mechanism by which the CP1 editing site catalyzes the hydrolysis of misactivated valine. Unlike proteolytic reactions where an amide bond must first be destabilized by the proteolytic enzyme, hydrolysis of the more labile ester and mixed anhydride moieties (within Val-AMP or Val-$tRNA^{Ile}$) is more likely to occur without an initial destabilization step. For this reason, it is possible that the IleRS editing site does not contain one or more specific residues that are directly involved in catalysis. Instead, it seems likely that T242 and N250 are important for binding both Val-AMP and Val-$tRNA^{Ile}$ to the editing site, possibly to accurately position them next to a catalytic water molecule. This hypothesis is supported by the observation that the T242P mutation, which should introduce unique structural constraints in the editing site, is more deleterious to accurate aminoacylation than the more subtle T242A perturbation.

In the linear sequence of IleRS, the CP1 editing site bisects the Rossman fold domain of the synthetic active site (*30*), and yet the substrate binding pockets of these two active sites are separated by approximately 25 Å (*17*). Thus, IleRS utilizes a tRNA-dependent translocation mechanism to shuttle misactivated valine from the synthetic active site to the hydrolytic editing site. The mechanism of translocation between these two sites remains unknown. The IleRS·$tRNA^{Ile}$ cocrystal structure demonstrated that the acceptor stem of $tRNA^{Ile}$ can partially unwind, thereby directing the 3′ end of the tRNA into the CP1 editing site (*18*). Although the tRNA was not aminoacylated in the cocrystal, misactivated Val-$tRNA^{Ile}$ most likely enters the editing site in an analogous fashion. The question remains, however, as to how Val-AMP is transferred from the synthetic active site to the editing site, in a $tRNA^{Ile}$-dependent manner.

An analysis of the primary sequence of the CP1 region of IleRS reveals that this insert shares similarity with two other proteins, valyl-tRNA synthetase (ValRS) and leucyl-tRNA synthetase (LeuRS) (*22*). These two enzymes and IleRS are more closely related to each other in evolution than to other class I aaRSs. Both ValRS and LeuRS are also reported to have tRNA-dependent editing reactions (*16*, *31*). Like IleRS, the Rossman-fold active sites of ValRS and LeuRS are each bisected by a conserved CP1 domain (*30*). It is these inserts that are homologous to the CP1 domain of IleRS and, by analogy, presumably contain the editing sites for ValRS and LeuRS. In fact, the CP1 domain of ValRS has been explicitly shown to deacylate Thr-$tRNA^{Val}$ (*16*), confirming this relationship between the editing sites of IleRS and ValRS. The similarities in CP1 domains between the three aminoacyl-tRNA synthetases suggest that CP1 was added in evolution prior to divergence of the three enzymes.

A portion of an alignment of CP1 from *E. coli* IleRS, ValRS and LeuRS is shown below:

| | | |
|---|---|---|
| IleRS | 240 | WTTTPWTLPAN |
| ValRS | 222 | ATTRPETLLGD |
| LeuRS | 248 | YTTRPDTFMGC |

Significantly, position T242 in IleRS (underlined) is conserved as a threonine in both ValRS and LeuRS. This conservation suggests that mutation of this position in either ValRS or LeuRS may lead to errors in aminoacylation similar to those observed in this work. Further, it raises doubt that T242 is directly involved in side chain recognition, because the IleRS editing site is specific for valine while the same site in ValRS accepts threonine and rejects valine. These observations suggest that even though each enzyme must hydrolyze different misactivated intermediates, they all use similar principles to maintain accurate tRNA aminoacylation.

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