An Aminoacyl-tRNA Synthetase with a Defunct Editing Site†

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ABSTRACT: Many aminoacyl-tRNA synthetases (aaRSs) contain two active sites, a synthetic site catalyzing aminoacyl-adenylate formation and tRNA aminoacylation and a second editing or proofreading site that hydrolyzes misactivated adenylates or mischarged tRNAs. The combined activities of these two sites lead to rigorous accuracy in tRNA aminoacylation, and both activities are essential to LeuRS and other aaRSs. Here, we describe studies of the human mitochondrial (hs mt) LeuRS indicating that the two active sites of this enzyme have undergone functional changes that impact how accurate aminoacylation is achieved. The sequence of the hs mt LeuRS closely resembles a bacterial LeuRS overall but displays significant variability in regions of the editing site. Studies comparing *Escherichia coli* and hs mt LeuRS reveal that the proofreading activity of the mt enzyme is disrupted by these sequence changes, as significant levels of Ile-tRNA^{Leu} are formed in the presence of high concentrations of the noncognate amino acid. Experiments monitoring deacylation of Ile-tRNA^{Leu} and misactivated adenylate turnover revealed that the editing active site is not operational. However, hs mt LeuRS has weaker binding affinities for both cognate and noncognate amino acids relative to the *E. coli* enzyme and an elevated discrimination ratio. Therefore, the enzyme achieves fidelity using a more specific synthetic active site that is not prone to errors under physiological conditions. This enhanced specificity must compensate for the presence of a defunct editing site and ensures translational accuracy.

The attachment of amino acids to tRNAs[1] by the aminoacyl-tRNA synthetases is a critical step in the translation of the genetic code (*1*−*3*). Aminoacylation must proceed with high efficiency and fidelity in order for proteins with defined sequences to be synthesized. Given the subtle structural differences among many of the 20 amino acids, the error-free conjugation of specific residues requires precise molecular recognition.

Many aaRSs use two active sites for amino acid discrimination that constitute a "double sieve" (*4*, *5*). In the first active site, amino acids are recognized, activated and converted to aminoacyl adenylates, and transferred to tRNA (Scheme 1). However, while amino acids larger than the cognate substrate can be excluded from this site by sterics, smaller amino acids can be bound, misactivated, and used erroneously to acylate tRNA. To combat this source of errors, some aaRSs employ a second active site that proofreads the products made by the activation site and hydrolytically edits molecules containing noncognate amino acids. Both pre-transfer editing of misactivated aminoacyl adenylates and post-transfer editing of misacylated tRNA can occur at this second active site (Scheme 1). Ablation of editing activity in many systems severely affects translational accuracy and interferes with cellular function (*6*−*8*).

The editing activities of several class Ia aaRSs are known to be performed by a insertion domain referred to as connective polypeptide 1 (CP1) (*9*). In IleRS, ValRS, and LeuRS, this insertion ranges from 250 to 275 amino acids in length and is highly conserved for a given enzyme across many species (*10*). Within these three class Ia enzymes, residues have been identified in CP1 that are critical for editing activity (*6*, *8*, *11*−*20*). In IleRS, mutations affecting post-transfer editing (*12*, *13*) and pre-transfer editing (*6*) have been reported, while in ValRS (*8*, *14*) and LeuRS (*15*−*20*), CP1 mutations are known that interfere with post-transfer editing. Studies of editing in class Ia aaRSs have focused on bacterial and yeast enzymes, and each of the systems investigated appears to possess this activity.

Recent structural and mutational studies of LeuRS editing centered on the enzymes from *Saccharomyces cerevisiae* cytoplasm (*19*), *Thermus thermophilus* (*19*), and *Escherichia coli* (*15*−*18*, *20*). All three LeuRSs display hydrolytic editing activity, and share highly conserved CP1 sequences. A crystal structure of the *T. thermophilus* LeuRS complexed with substrate analogues localized to the editing site revealed the precise location of the binding site for misacylated amino acids (*19*). Mutations made within this region in *S. cerevisiae* (*19*), *T. thermophilus* (*19*), and *E. coli* LeuRS can attenuate editing of noncognate substrates (*15*−*18*, *20*) or induce the editing of the cognate amino acid attached to tRNA (*16*).

The human mitochondrial (hs mt) LeuRS presents a novel system for the analysis of editing activity. Whether proofreading activity is generally conserved for human aaRSs is not known, and editing has not been examined in any mitochondrial system. In addition, the hs mt LeuRS has received significant attention because its cognate

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[1] Abbreviations: hs mt, human mitochondrial; tRNA, transfer ribonucleic acid; aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; PAGE, polyacrylamide gel electrophoresis; WT, wild-type; AA, amino acid.

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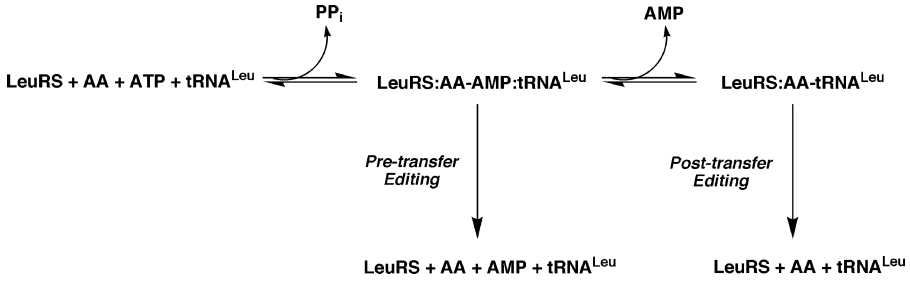
Scheme 1: Pre- and Post-Transfer Editing Commonly Exhibited by Class 1a aaRSs



tRNA$^{Leu(UUR)}$ is the site of a number of disease-related mutations (*21*, *22*), and in many cases the deleterious effects of the pathogenic substitutions have been linked to attenuated or erroneous aminoacylation (*23*−*25*), in addition to other pathways. The sequence of the hs mt LeuRS, which is distinct from the enzyme functioning in human cytoplasm, is >35% identical to many bacterial LeuRSs (*26*) (see Figure 1 in Supporting Information for full alignment), but displays very low homology to cytoplasmic LeuRSs from eukaryotes, consistent with its probable origin as a bacterial enzyme. The highest levels of identity between prokaryotic LeuRSs and the hs mt LeuRS are found in the N-terminal domains, and in particular, within the regions of the enzyme that comprise the amino acid and ATP binding pockets within the activation site.

We report here on the unique features of the hs mt LeuRS that allow this enzyme to achieve accurate aminoacylation. The activity of the editing site within the hs mt LeuRS was investigated, and it was discovered that this LeuRS does not exhibit the pre- or post-transfer editing activity observed with the bacterial homologue. However, the hs mt LeuRS displays a high level of selectivity during amino acid activation, and is more specific for leucine compared to *E. coli* LeuRS. Moreover, the hs mt enzyme displays very weak affinity for isoleucine. Thus, the concentrations of the noncognate amino acid required for appreciable levels of misactivation are elevated beyond physiological levels. It appears that the sequence of the hs mt LeuRS has accumulated substitutions rendering the editing site defunct, but the heightened specificity of this enzyme during amino acid activation may provide sufficient accuracy during aminoacylation to minimize errors. This enzyme therefore represents a unique case of a LeuRS that relies on a single sieve for high-fidelity aminoacylation.

MATERIALS AND METHODS

Cloning and Preparation of tRNA Constructs. Wild-type hs mt tRNA$^{Leu(UUR)}$ and *E. coli* tRNA$^{Leu(CUN)}$ were prepared as described (*24*). Plasmids were harvested from XL1-Blue competent cells (Stratagene) and digested with *Mva1* (Ambion) to generate the 3′ CCA end. The digested DNA was then phenol/chloroform extracted (pH 8, Sigma), ethanol precipitated and resuspended in distilled H_2O. The DNA was furthered purified using G-25 columns (Amersham Pharmacia). Transcription reactions were performed using template DNA (200−400 μg/mL), T7 RNA polymerase (overexpressed in *E. coli*), RNAsin (400 units/mL, Promega), 40 mM Tris-HCl (pH 8), 10 mM NaCl, 2 mM spermidine, 20 mM $MgCl_2$, 4 mM NTPs and 5 mM dithiothreitol. Samples were incubated at 37 °C for 4 h, with the addition of a second aliquot of polyermase after 2 h. The DNA template was then digested with DNase I (60 units/mL, Takara) for 30 min. RNA products were extracted with 5:1 phenol/chloroform (pH 4.7, Sigma) and ethanol precipitated. Transcription products were further purified by 12% denaturing PAGE using 0.5X TBE buffer (45 mM Tris base/45 mM boric acid/1mM EDTA) for 4 h. Purified transcripts were recovered by electroelution, and were ethanol precipitated. tRNA was resuspended in 0.5X TE (5mM Tris-HCl (pH 8), 0.5 mM EDTA). All solutions were prepared with diethyl pyrocarbonate (DEPC) treated water.

Absorbance at 260 nm was used to quantify the concentration of tRNA in solution. Values were obtained by applying an extinction coefficient of 895,000 M^{-1} (mononucleotide) cm^{-1} (hs mt tRNA^{Leu}) and 905,000 M^{-1} cm^{-1} (*E. coli* tRNA^{Leu}) (extinction coefficient obtained from calculator available from http://www.genscript.com). tRNA samples were annealed with incubation at 70 °C for 5 min in distilled water followed by addition of $MgCl_2$ (10 mM) and immediate cooling on ice for at least 20 min.

Preparation of hs mt, WT E. coli, and E. coli T252Y LeuRS. Hs mt LeuRS was expressed and purified as described (*24*, *26*). *E. coli* LeuRS and mutant T252Y were purified from SG13009 cells carrying the pREP4 repressor plasmid as described (*20*); both plasmids were provided by D. Tirrell, Caltech, Pasadena, CA. After cell lysis in a French press, the enzymes were purified using Ni−NTA agarose (Qiagen). The purity of the protein was confirmed by SDS−PAGE. Enzyme concentrations were determined by Bradford protein assay (Biorad).

Isolation of hs mt tRNA^{Leu} and E. coli tRNA^{Leu} Misacylated with Isoleucine. tRNA samples were annealed as described above. Folded hs mt tRNA^{Leu} was misaminoacylated with 10 μM [3,4,5-^{3}H] isoleucine (Amersham Pharmacia) in a reaction containing 5 μM hs mt LeuRS, 5 μM hs mt tRNA^{Leu}, 500 μM isoleucine, 50 mM HEPES (pH 7.0), 0.2 mg/mL bovine serum albumin, 25 mM KCl, 100 μM spermine, 7 mM $MgCl_2$, 2.5 mM ATP, and 10% (v/v) dimethyl sulfoxide (DMSO) (*27*). The *E. coli* tRNA^{Leu} was mischarged in the same reaction mixture without DMSO and with 5 μM *E. coli* T252Y LeuRS and 2 μM *E. coli* tRNA^{Leu}. The reactions were incubated at 37 °C for 1 h, and then quenched by lowering the pH to 6.0 with 0.2% acetic acid (*17*). The mischarged tRNA was concentrated by butanol extraction (1:1) and then immediately extracted using 5:1 phenol /chloroform (pH 4.7, Sigma) and ethanol precipitated. The RNA pellet was washed two times with cold 80% ethanol, resuspended in 20 mM potassium phosphate (pH 5.0) and stored at −20 °C. Prior to storage and subsequent use in

deacylation assays, mischarged tRNA was quantitated by scintillation counting after TCA precipitation.

Deacylation of hs mt Ile-tRNA^{Leu} and E. coli Ile-tRNA^{Leu}. Hs mt Ile-tRNA^{Leu} (250 nM) was incubated at 37 °C with 50 mM HEPES (pH 7.0), 0.2 mg/mL bovine serum albumin, 25 mM KCl, 100 μM spermine, 7 mM $MgCl_2$ and 2 μM hs mt or *E. coli* LeuRS. Assays with *E. coli* Ile-tRNA^{Leu} (200 nM) were performed under the same conditions as mentioned above. The amount of deacylation was quantitated by scintillation counting.

Cross-Aminoacylation and Misaminoacylation Assays. tRNA samples were annealed as described above. Cross-species leucylation assays were performed at 37 °C in reaction mixtures containing 50 mM HEPES (pH 7.0), 0.2 mg/mL bovine serum albumin, 25 mM KCl, 100 μM spermine, 7 mM $MgCl_2$, 100 μM leucine, 5 μM [3,4,5-^{3}H] leucine (Perkin-Elmer), 3 μM hs mt tRNA^{Leu} or *E. coli* tRNA^{Leu} and 20 nM hs mt or *E. coli* LeuRS. Aliquots (2 μL) of the reaction mixture were precipitated on prewet and dried Whatman circles with 5% TCA, washed three times with 5% TCA, then soaked in ethanol before drying. The level of aminoacylation of the tRNA was determined by scintillation counting.

Misaminoacylation assays were performed as described above, except that each reaction contained 13.4 μM [3,4,5-^{3}H] isoleucine, 500 μM isoleucine, 5 μM hs mt tRNA^{Leu} or *E. coli* tRNA^{Leu}, and 1 μM hs mt or *E. coli* LeuRS.

ATP-PP$_i$ Exchange Assay. Amino acid activation by hs mt and *E. coli* LeuRS was analyzed at 37 °C in reaction mixtures containing 100 mM Tris-HCl (pH 7.5), 10 mM potassium fluoride (Labchem), 5 mM $MgCl_2$, 25 mM ATP, 7 mM 2-mercaptoethanol, 1 mg/mL bovine serum albumin, 6.6 μM [^{32}P]-PP$_i$, 500 nM hs mt LeuRS and 75 mM leucine, isoleucine, valine, threonine, methionine, serine, alanine or glycine. For *E. coli* LeuRS, 10 nM enzyme was used with 15 mM amino acid. Aliquots (45 μL) of the reaction were removed and quenched in 450 μL of 6% activated charcoal, 0.3% HCl, 0.12 M NaPP$_i$, and 3.8% $HClO_4$. The quench solution was then transferred to a screening column (Fisher). The charcoal was washed three times with quench buffer (0.2 M NaPP$_i$, and 7% $HClO_4$). The amount of [^{32}P]-PP$_i$ converted into [^{32}P]-ATP was quantified by scintillation counting of the charcoal.

Kinetic parameters for leucine activation were determined using 20 nM hs mt or *E. coli* LeuRS and concentrations of leucine ranged from 10 μM − 5000 μM. For isoleucine activation, 200 nM hs mt or *E. coli* LeuRS were used. The concentrations of isoleucine ranged from 1 mM − 140 mM (hs mt LeuRS) and 0.1 mM − 7 mM (*E. coli* LeuRS). The data represents the average of at least three determinations.

Analysis of ATP Hydrolysis. ATP hydrolysis was measured using a TLC-based method adapted from that described by Wolfson and Uhlenbeck (*28*). Hs mt LeuRS (10 μM) was incubated with 50 mM HEPES (pH 7.0), 0.2 mg/mL bovine serum albumin, 25 mM KCl, 100 μM spermine, 7 mM $MgCl_2$, 20 μg/mL inorganic pyrophosphatase (Roche Molecular Biochemicals), 1 mM ATP, 1 μCi of [α-^{32}P]-ATP (6000 Ci/mmol, Perkin-Elmer), 625 μM isoleucine and 10 μM tRNA^{Leu} for 1 h at 37 °C in a total volume of 8 μL. At each time point, 0.5 μL was spotted on a 10 cm polyethyleneimine cellulose thin-layer chromatography (TLC) plates (J. T. Baker). Plates were developed in 0.5 M lithium chloride and conversion of [α-^{32}P]-ATP to [α-^{32}P]-AMP was visualized with a Bio-Rad Molecular Imager FX Pro-Plus phosphoimager. ATPase assays for *E. coli* LeuRS (10 μM) with *E. coli* tRNA^{Leu} (10 μM) were performed under the same conditions.

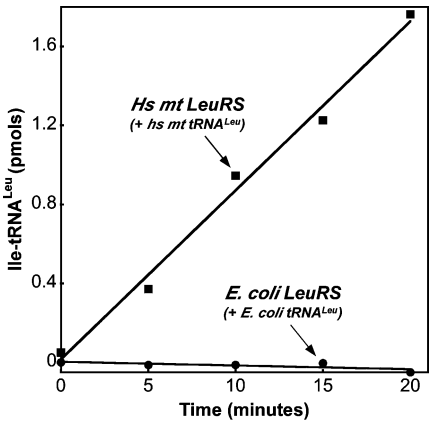


FIGURE 1: Mischarging of tRNA^{Leu} with isoleucine by LeuRS. Shown is the misaminoacylation activity of *E. coli* and hs mt LeuRS with their respective cognate tRNAs under identical conditions (1 μM LeuRS, 5 μM tRNA^{Leu}, 513 μM isoleucine, 37 °C, pH 7). Data shown represent average values obtained from ≥3 trials.

RESULTS

Misacylation by the hs mt LeuRS. The misacylation of amino acid substrates and hs mt tRNA^{Leu} was monitored to determine whether the hs mt LeuRS was able to effectively edit its charged products. To identify the amino acids that were most efficiently misactivated, a series of structurally similar amino acids was tested (see Supporting Information Figure 2). Initial rates of ATP−PP$_i$ exchange were measured to gauge the reactivities of different substrates. Aside from leucine, isoleucine was the most efficiently activated, followed by valine, methionine, threonine, serine, alanine, and glycine. The substrate specificity observed for hs mt LeuRS was identical to that of *E. coli* LeuRS, although, as previously reported by Spremulli and co-workers (*26*), the leucine activation activity of the human enzyme was reduced relative to the bacterial enzyme.

The hs mt LeuRS displays significant misacylation of tRNA^{Leu} in the presence of isoleucine (Figure 1). Misacylated tRNA is not detected when the *E. coli* LeuRS is analyzed under the same conditions. When high concentrations of amino acids are present (0.5 mM), aminoacylation of the hs mt tRNA^{Leu} with isoleucine is reduced only ∼250 times relative to leucine, but for the *E. coli* tRNA and enzyme, we estimate that cognate and noncognate charging differ by a much larger factor (>3000).

Deacylation of Ile-tRNA^{Leu} by E. coli and hs mt LeuRS. To determine whether the accumulation of Ile-tRNA^{Leu} observed with hs mt LeuRS reflected the inability of the enzyme to proofread noncognate amino acids, the enzymatic deacylation of the mischarged substrate was monitored (Figure 2). The *E. coli* LeuRS and tRNA^{Leu} was used as a control to evaluate the integrity of the human tRNA substrate and cross-species activity.

Unlike the other LeuRS paralogs studied to date (*15*−*20*), the hs mt LeuRS does not deacylate its cognate tRNA mischarged with isoleucine (Figure 2A). Interestingly, the presence of the enzyme inhibits the spontaneous deacylation

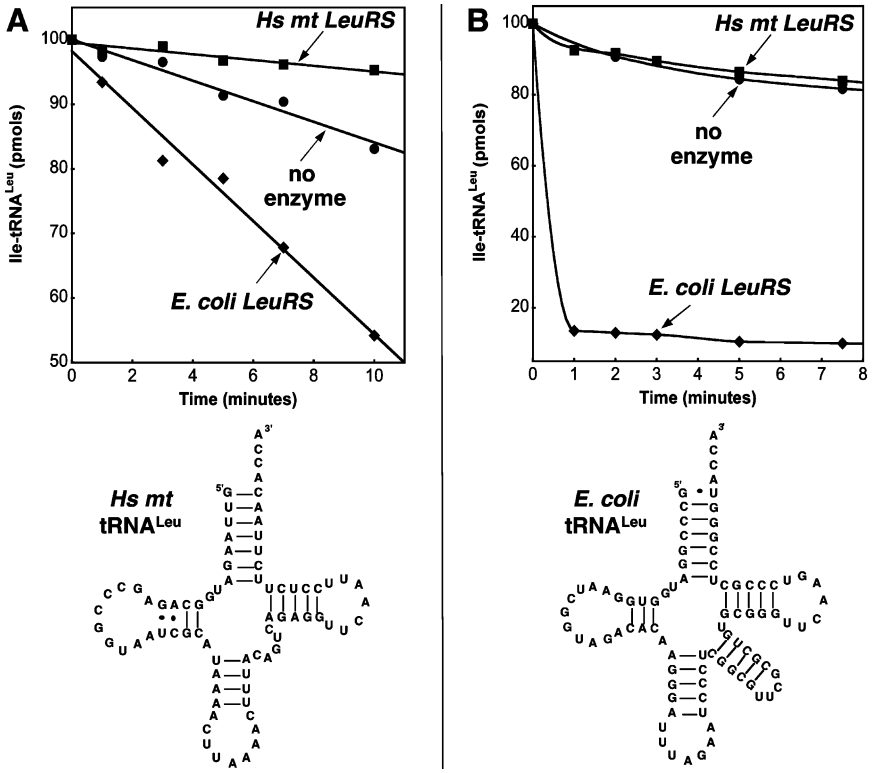


FIGURE 2: Deacylation of Ile-tRNA^{Leu} by *E. coli* and hs mt LeuRS. WT *E. coli* and hs mt LeuRS were assayed for their ability to enzymatically deacylate (A) hs mt Ile-tRNA^{Leu} (250 nM) or (B) *E. coli* Ile-tRNA^{Leu} (200 nM) under identical conditions (2 μM LeuRS, 37 °C, pH 7). The structures of the two tRNAs are shown for reference. Data shown represent average values obtained from ≥3 trials.

that occurs in aqueous solution. Mischarged *E. coli* tRNA^{Leu} was also tested as a substrate, as the hs mt LeuRS aminoacylates this tRNA with high efficiency (Figure 3B). Deacylation did not occur with this tRNA (Figure 2B).

In contrast, *E. coli* LeuRS catalyzes the deacylation of both the *E. coli* and hs mt Ile-tRNA^{Leu} (Figure 2A and 2B). This result is significant for several reasons. Deacylation of the hs mt Ile-tRNA^{Leu} provides confirmation that this tRNA is an active substrate. While the hs mt tRNA transcript is efficiently charged by the cognate LeuRS, its structure is known to be thermodynamically unstable and loosely structured (*29*). Therefore, the lack of editing observed with the hs mt LeuRS could indicate that this activity is strongly dependent on the presence of a folded structure. However, the observation of deacylation of this tRNA by *E. coli* LeuRS confirms that an enzyme with an active editing site can process this substrate, albeit with reduced efficiency. The reactivity of the *E. coli* LeuRS with hs mt Ile-tRNA^{Leu} is also noteworthy because the bacterial enzyme does not aminoacylate this tRNA (Figure 3A). Therefore, while amino acids cannot be charged onto the hs mt tRNA by the *E. coli* synthetase, they can be removed. This observation highlights the specificity of the editing site toward the amino acid rather than the tRNA.

ATP Hydrolysis Activity of E. coli and hs mt LeuRS. To assess whether the hs mt LeuRS exhibits pre-transfer editing activity (i.e., the ability to hydrolyze misactivated isoleucine-AMP), we monitored ATP turnover in the presence of isoleucine for the hs mt and *E. coli* LeuRS (Figure 4). This assay would typically report on both pre-transfer and post-transfer editing, but given the fact that deacylation was shown not to occur for hs mt LeuRS, the experiments performed provide information on the ability of the enzyme to process Ile-AMP via hydrolytic editing alone.

The results obtained in the ATP hydrolysis experiment mirror the deacylation results described above. The hs mt LeuRS does not exhibit increased ATP consumption in the presence of tRNA^{Leu} and isoleucine, indicating that the enzyme does not edit adenylates containing misactivated amino acid. The *E. coli* LeuRS does promote tRNA-dependent ATP hydrolysis in the presence of isoleucine, which may reflect pre-transfer editing in addition to the post-transfer deacylation activity described above and reported elsewhere (*15*−*20*).

Specificity of Amino Acid Activation by hs mt LeuRS. Given the pronounced difference in the activities of the *E. coli* and hs mt LeuRS editing sites, we wondered whether the activation sites of these enzymes had unique properties. To compare the affinities and specificities of *E. coli* and hs mt LeuRS for cognate and noncognate amino acids, pyrophosphate exchange was used to extract kinetic parameters for the reaction of LeuRS with leucine and isoleucine (Table 1). These experiments revealed several key differences in the amino acid activation sites for the two systems. Activation of both leucine and isoleucine by hs mt LeuRS proceeds with lower overall efficiencies relative to *E. coli* LeuRS. Both catalytic turnover and amino acid binding appear to be affected in the hs mt enzyme. Notably, the activation of isoleucine is more strongly attenuated than leucine. The k_{cat}/K_m values for *E. coli* relative to hs mt LeuRS vary by a factor of 50 for leucine, and a factor of 100 for isoleucine. This is in part due to a high K_m value for isoleucine with hs mt LeuRS. The apparent binding affinity for isoleucine is 50 times weaker for the hs mt LeuRS relative to *E. coli* LeuRS.

The main conclusion drawn from the analysis of the efficiency of amino acid activation is that the hs mt LeuRS exhibits enhanced discrimination of leucine from isoleucine. The discrimination ratio for hs mt LeuRS is 3000, while for

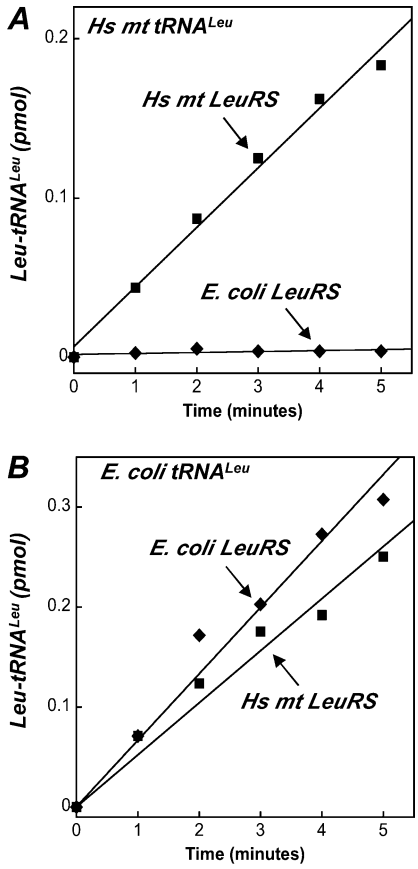


FIGURE 3: Cross-species aminoacylation of (A) hs mt $tRNA^{Leu}$ (3 μM) or (B) *E. coli* $tRNA^{Leu}$ (3 μM) with leucine by *E. coli* and hs mt LeuRS (20 nM). Assays were performed at 37 °C at pH 7. Data shown represent average values obtained from ≥3 trials. The experiments described were carried out with the *E. coli* $tRNA^{Leu(CUN)}$ and hs mt $tRNA^{Leu(UUR)}$ isoacceptors. Comparable results were obtained with the hs mt $tRNA^{Leu(CUN)}$, but the $tRNA^{Leu(UUR)}$ was selected as the focus of these studies because its structure is more thoroughly characterized (*29*).

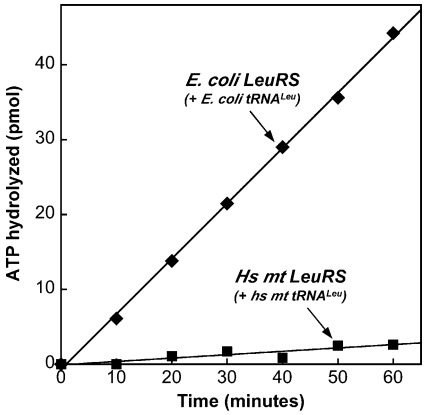


FIGURE 4: Total editing activities of *E. coli* and hs mt LeuRS as measured by the hydrolysis of ATP to AMP. Editing of isoleucine (625 μM) by *E. coli* and hs mt LeuRS (10 μM) was assayed with the respective cognate tRNA (10 μM) at 37 °C, pH 7. The data shown was corrected for background ATP hydrolysis that occurs in the absence of amino acid. Data shown represent average values obtained from ≥3 trials.

E. coli LeuRS, it is 1600 (Table 1). Moreover, these ratios are even more disparate when the relative availabilities of different amino acids in bacteria and mitochondria are considered. According to published reports, the concentration of isoleucine is 5-fold higher than leucine in *E. coli* (*30*), while in mammalian mitochondria, leucine is present at levels that exceed those of isoleucine by a factor of 2 (*31*). If these values are taken into account, the discrimination ratio for the *E. coli* LeuRS drops to only 320, whereas the human enzyme would be able to achieve higher specificity with an adjusted discrimination ratio of 6000.

Table 1: Kinetic Parameters for the Activation of Leucine and Isoleucine by *E. coli* and hs mt LeuRS[a]

	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}\,\mu M^{-1}$)	discrimination factor
E. coli LeuRS				
leucine	0.015 ± 0.002	11.0 ± 0.4	0.8	1600
isoleucine	0.25 ± 0.05	0.10 ± 0.2	0.0005	
hs mt LeuRS				
leucine	0.13 ± 0.02	2.0 ± 0.1	0.015	3000
isoleucine	14 ± 2	0.070 ± 0.004	0.000005	

[a] Kinetic parameters were determined at pH 7.5 and 37 °C.

DISCUSSION

An Inactive Editing Site within hs mt LeuRS. The results reported here demonstrate that the hs mt LeuRS does not display the type of editing activity observed for other LeuRSs (*15*−*20*). Furthermore, these data suggest the hs mt LeuRS achieves aminoacylation fidelity solely via its synthetic active site, a mechanism that distinguishes this enzyme from other bacterial and eukaryotic LeuRSs. Many of the class Ia aaRSs are confronted with a difficult molecular recognition problem during amino acid substrate selection. IleRS (*6*, *11*−*13*), ValRS (*8*, *14*), and LeuRS (*15*−*20*) must discriminate cognate amino acids from isomeric structures or substrates with other subtle structural differences. All three of these aaRSs, therefore, appear to have adopted a second active site to impart higher fidelity to aminoacylation. Prior to this report, proofreading activity in LeuRSs was thought to be universally conserved, as editing activity is well-documented for *E. coli* (*15*−*18*, *20*), *T. thermophilus* (*19*), and *S. cerevisiae* (*19*) LeuRS. All of these enzymes efficiently deacylate tRNA mischarged with a variety of noncognate amino acids, and ATP hydrolysis activity that may represent aminoacyl adenylate processing has been reported for the *E. coli* enzyme (*32*).

A crystallographic structure of the *T. thermophilus* (*19*) enzyme complexed with small molecule inhibitors bound to the editing site provided precise information on the location of residues that comprise the binding pocket for the noncognate amino acid. An alignment of a series of bacterial LeuRSs and the *S. cerevisiae* LeuRS shows that there is significant conservation within this part of CP1, particularly at residues that have been demonstrated to be essential for editing (Figure 5A). Interestingly, the hs mt LeuRS (and other mt analogues) display significant sequence variations in this same region. While other portions of the mt LeuRSs, e.g. the active site (see Figure 5B), are almost identical to the bacterial LeuRSs, the editing site appears to have undergone substantial divergence away from the bacterial sequence. The *Caenorhabditis elegans* mt and hs mt LeuRS both display significant but divergent variability in the regions of the sequence comprising the editing site in bacterial LeuRSs. This trend indicates that both species have accumulated sequence changes independently, and at least in the case of the hs mt LeuRS, it appears that these sequence changes have deactivated the editing site.

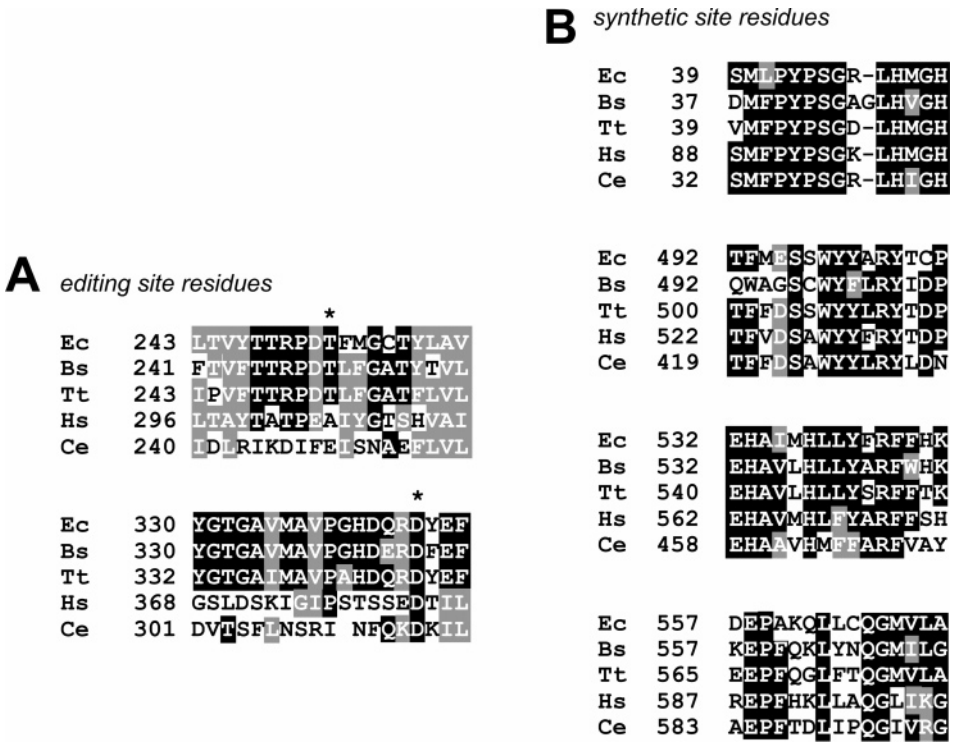


FIGURE 5: Multiple sequence alignments of the portions of the (A) editing and (B) active sites of LeuRSs from *E. coli* (Ec), *T. thermophilus* (Tt), *Bacillus subtilus* (Bs), human mitochondria (Hs), and *C. elegans* mitochondria (Ce). Fragments shown were selected based on the binding pockets for noncognate (editing site, A) and cognate amino acids (synthetic site, B) revealed by the structure of *T. thermophilus* LeuRS. Residues shaded in black are conserved for at least three sequences, whereas residues shaded in gray reflect residues that vary but have similar side chains. Asterisks represent residues shown to be important for editing function of *E. coli* LeuRS enzyme. The sequence source was http://au.expasy.org/ and alignment was generated using CLUSTALW.

Enhanced Discrimination of Amino Acids by hs mt LeuRS. The fact that the editing activities observed for the hs mt and *E. coli* LeuRSs inversely mirror the degree of amino acid specificity in the two enzymes suggests that the burden of amino acid discrimination is differently distributed between the editing and activation sites of the two systems. The hs mt LeuRS appears to compensate for the absence of editing ability with an elevated level of substrate specificity during amino acid activation (Table 1). Relative to the bacterial LeuRS, the hs mt enzyme exhibits less efficient amino acid activation, which primarily reflects higher K_m values for amino acid substrates. However, although it is less active, the hs mt LeuRS more accurately discriminates leucine from isoleucine. While the K_m for leucine is 0.13 mM, the K_m for isoleucine is 14 mM. This difference, along with more disparate k_{cat} values for the noncognate and cognate amino acids, makes the mt enzyme more accurate. Interestingly, the hs mt LeuRS discrimination ratio (describing the differential activation of leucine versus isoleucine) is 3000. Previous studies of protein synthesis error rates have prompted the prediction that editing function will exist for aaRSs with discrimination ratios <3000 in order for translational accuracy to be maintained at observed levels (*33*−*35*). The absence of this activity for the hs mt LeuRS, which has a discrimination ratio of 3000 from its active site alone, is consistent with this prediction. It would appear that high amino acid specificity at the activation site makes editing unnecessary, at least in the intramitochondrial environment.

Moreover, the low affinity of hs mt LeuRS for its amino acid substrates may assist the enzyme in achieving fidelity. While exact concentrations of amino acids in human mitochondria have not been documented (only relative ratios are reported) (*31*), in bacterial cells levels of individual amino acids do not typically exceed 1 mM (*30*). Given the high amino acid K_m values we measured, the activation of isoleucine by the hs mt LeuRS would be a low-frequency event. Additionally, levels of leucine (present at a concentration of 10 μM in *E. coli* (*30*)) are 2-fold higher than isoleucine in mitochondria (*31*), indicating that reactions involving the noncognate substrate would be very inefficient. The higher specificity and lowered efficiency of the hs mt LeuRS relative to *E. coli* LeuRS may reflect the optimization of the protein's function within mitochondria. Many mitochondrial enzymes exhibit lower levels of activity than their bacterial or cytoplasmic counterparts (*26*, *36*−*38*), indicating that efficiency of biological processes in this organelle may be reduced without severe cellular ramifications.

This report documents the second human aaRS that has lost editing activity, as a recent study of the human ProRS revealed that this enzyme cannot deacylate mischarged tRNA (*39*). In addition, the recent discoveries of editing enzymes that deacylate mischarged tRNAs *in trans* present the possibility that an external factor is responsible for proofreading hs mt $tRNA^{Leu}$ (*40*−*42*). However, the hs mt LeuRS may not require any editing activity because the precision of its active site engenders high aminoacylation specificity. This system constitutes an example of a class Ia aaRS that needs only a single sieve for accurate aminoacylation.

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SUPPORTING INFORMATION AVAILABLE

Sequence alignments and amino acid activation data. This material is available free of charge via the Internet at http://pubs.acs.org

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