Aminoacyl-tRNA synthetases are a family of enzymes that are responsible for translating the genetic code in the first step of protein synthesis. Some aminoacyl-tRNA synthetases have editing activities to clear their mistakes and enhance fidelity. Leucyl-tRNA synthetases have a hydrolytic active site that resides in a discrete amino acid editing domain called CP1. Mutational analysis within yeast mitochondrial leucyl-tRNA synthetase showed that the enzyme has maintained an editing active site that is competent for post-transfer editing of mischarged tRNA similar to other leucyl-tRNA synthetases. These mutations that altered or abolished leucyl-tRNA synthetase editing were introduced into complementation assays. Cell viability and mitochondrial function were largely unaffected in the presence of high levels of non-leucine amino acids. In contrast, these editing-defective mutations limited cell viability in Escherichia coli. It is possible that the yeast mitochondria have evolved to tolerate lower levels of fidelity in protein synthesis or have developed alternate mechanisms to enhance discrimination of leucine from non-cognate amino acids that can be misactivated by leucyl-tRNA synthetase.

Aminoacyl-tRNA synthetases are an ancient family of enzymes that are responsible for carrying out the two-step aminoacylation reaction (1). In the first step, an amino acid is condensed with a single molecule of ATP to form an aminoacyl-adenylate intermediate. The activated amino acid is then transferred to the 3′-terminal adenosine of its cognate isoacceptor tRNA (2, 3). Inaccurate aminoacylation could yield infidelities during protein synthesis that would be expected to be detrimental for cell viability and survival (4).

Linus Pauling had originally predicted that the rate of misactivation of isosteric substrates that differed by one methyl group (for example, alanine and glycine or isoleucine and valine) would be as high as 1 in 5 (5). However, the in vivo rate of valine misincorporation for isoleucine into cellular proteins was measured to be as low as 1 in 3000 (6). This increased fidelity has been attributed to a double sieve mechanism that enhances discrimination and accurate selection of the correct amino acids (7–9). The canonical aminoacylation core acts as a coarse sieve where activation of the cognate amino acid as well as smaller non-cognate amino acids occurs. A second fine sieve excludes the correctly charged amino acid and hydrolyzes misacylated tRNA.

The homologous LeuRS, isoleucyl-tRNA synthetase (IleRS), and valyl-tRNA synthetase (ValRS) contain these two active sites to enhance fidelity (10–13). Each of their amino acid editing active sites is located in a discretely folded domain of ~200 amino acids called CP1 (14–16). The CP1 domain is inserted into the main body of the enzyme via two β-strand linkers (10, 12, 17, 18).

The editing mechanisms of ValRS and IleRS primarily target threonine and valine, respectively, in the cell (11, 19–22). However, LeuRS misactivates many non-leucine amino acids, including isoleucine, valine, methionine, and also structurally similar metabolic cellular intermediates (e.g. norleucine, norvaline, α-aminobutyrate, and homocysteine (23–27)) as well as synthetic hydroxylated (24) and fluorinated (28) leucine derivatives. Biochemical investigations and co-crystal structures of LeuRSs have identified a number of key elements of the LeuRS amino acid editing active site (13, 18, 27, 29–31). A conserved threonine-rich region within the CP1 domain comprises a portion of the editing active site pocket in Escherichia coli LeuRS and confers specificity and activity (15, 27, 32–34). Significantly, a key specificity discriminator at a conserved threonine in the editing active site blocks cognate leucine from binding (15) while allowing this wide array of amino acids to bind for hydrolytic editing.

LeuRS shares a universally conserved aspartic acid that is also found in the CP1 domains of IleRS and ValRS. The aspartate forms a hydrogen bond with the amino moiety of the bound amino acid and is essential for hydrolytic editing. Mutations of this conserved aspartate abolish the editing activity of LeuRS, IleRS, and ValRS in vitro (13, 35, 36).

We hypothesized that cell viability and growth would be significantly affected by editing-defective LeuRS enzymes. Statistical protein mutations that are introduced via mischarged tRNA\(^\text{Leu}\) would be expected to alter or abolish the function of critical proteins. Our results show that amino acid editing is critical to bacterial survival. In contrast, the yeast mitochondrion appears to tolerate LeuRS infidelities. Thus, different organisms have varied thresholds for fidelity. It is also possible that these organisms have evolved alternate mechanisms to enhance fidelity that extend beyond the LeuRS CP1 domain.
Editing of Yeast Mitochondrial Leucyl-tRNA Synthetase

ExPERIMENTAL PROCEDURES

Materials—Oligonucleotides were obtained from MWG Biotech (High Point, NC). All 3H-labeled amino acids were purchased from Amersham Biosciences. Restriction endonucleases were acquired from Promega (Madison, WI), except BstNI, which was from New England Biolabs, Inc. (Beverly, MA). Cloned Pfu DNA polymerase and commercially mixed dNTPs were obtained from Stratagene (La Jolla, CA). The E. coli temperature-sensitive strain K1231 (F- thy-35, str-120, leu351) was obtained from the E. coli Genetic Stock Center (Yale University, New Haven, CT) (37). The plasmid pGP1-2 (38) encoding the T7 RNA polymerase was a gift from Dr. Tracy Palmer (University of East Anglia).

Site-directed Mutagenesis—The plasmid pYM3–17 (39) expressing the wild-type yeast mitochondrial LeuRS was used as a template to introduce mutations including T263V/T264V (TT/VV) for overexpression and purification of each protein for in vitro studies. Likewise, the plasmid ymLEURST (40), encoding the wild-type yeast mitochondrial LeuRS, was used as a template to generate the same set of mutations, T263V/T264V (TT/VV), R265A (pVK064), and D357A (pVK078) for overexpression and purification of each protein for in vitro studies. The reactions were then restriction-digested with 20 units of restriction enzyme and 1 µl of T4 DNA ligase. The ligated DNA was transformed into the temperature-sensitive strain K1231 (F- thy-35, str-120, leu351) which was from New England Biolabs, Inc. (Beverley, MA). The overnight culture was transferred to 500 ml of LB-Amp/Cm and grown overnight at 37 °C. The reaction mixture was incubated for 3 h at 42 °C followed by a second addition of 500 µl T7 RNA polymerase and then incubated for an additional 3 h at 42 °C.

The RNA product was purified on a 10% polyacrylamide gel (19:1) 8 wv urea gel. The RNA band was detected via UV shadowing, excised from the gel, and extracted overnight in 0.5 M NH4OAc and 1 mM EDTA, pH 8.0, at 37 °C. The supernatant was collected and the extraction repeated two more times. The RNA was then purified by butanol extraction, and a total of 1 µg/µl glycogen was added to the concentrated RNA followed by ethanol precipitation. The RNA pellet was washed twice in 70% ethanol, dried, and then resuspended in 100 µl of nuclease-free water (Ambion, Austin, TX). Purified tRNALeu was denatured at 80 °C for 1 min followed by the addition of 1 mM MgCl2 and quick cooling on ice. The concentration of the tRNA was determined by measuring the absorbance at 260 nm using a calculated extinction coefficient of 87,500 L·mol⁻¹·cm⁻¹ (43). Plateau values for fully charged tRNALeu transcript indicated that ~55% of the tRNA pool was competent for leucylation by yeast mitochondrial LeuRS.

Aminoacylation Assays—Aminoacylation reactions contained 60 mM Tris, pH 7.5, 10 mM MgCl2, 150 mM KCl, 1 mM dithiothreitol, 0.8 µM yeast mitochondrial tRNALeu, 22 µM [3H]leucine (152 Ci/mmol), and 50 µM enzyme. The addition of KCl stimulates the enzymatic activity of yeast mitochondrial LeuRS.2 Misaminoacylation assays were carried out similarly, except 22 µM [3H]isoleucine (92 Ci/mmol) and 1 µM enzyme were used. The reactions were initiated with 4 mM ATP and carried out at room temperature. At specific time points, 5 µl of the reaction were quenched on Whatman grade 3 filter pads that were pre-equilibrated with HA-1. The lysate was separated from the resin by low speed centrifugation in a clinical centrifuge. The resin was then washed with HA-2 (20 mM NaPi, 10 mM Tris, pH 7.0, 500 mM NaCl, 5 mM imidazole, and 5% glycerol) six times for 10 min each. The N-terminal His6-tagged LeuRS was eluted using 1 ml of HA-1 that contained 200 mM imidazole. Protein was concentrated using a centricon-50 (Amicon, Bedford, MA) and its concentration measured via the Bio-Rad protein assay according to the commercial protocol.

Preparation of tRNAleu—The plasmid pymtDNAleu1 containing the gene for yeast mitochondrial tRNALeu was digested for 6 h at 60 °C with 25 units of BstNI and then used as the template for in vitro transcription (41, 42). A 1 ml reaction contained 450 µg of plasmid template, 40 mM Tris, pH 8.0, 30 mM MgCl2, 5 mM dithiothreitol, 0.01% Triton X-100, 50 µg/ml bovine serum albumin, each dNTP at 4 mM, 80 mg/ml polyethylene glycol 8000, 0.02 units/µl RNase inhibitor (Eppendorf, Hamburg, Germany), 2 mM spermidine, 0.01 mg/ml E. coli pyrophosphatase (Sigma), and 500 nM T7 RNA polymerase. The reaction mixture was incubated for 3 h at 42 °C followed by a second addition of 500 nM T7 RNA polymerase and then incubated for an additional 3 h at 42 °C.

The RNA product was purified on a 10% polyacrylamide gel (19:1) 8 wv urea gel. The RNA band was detected via UV shadowing, excised from the gel, and extracted overnight in 0.5 M NH4OAc and 1 mM EDTA, pH 8.0, at 37 °C. The supernatant was collected and the extraction repeated two more times. The RNA was then purified by butanol extraction, and a total of 1 µg/µl glycogen was added to the concentrated RNA followed by ethanol precipitation. The RNA pellet was washed twice in 70% ethanol, dried, and then resuspended in 100 µl of nuclease-free water (Ambion, Austin, TX). Purified tRNALeu was denatured at 80 °C for 1 min followed by the addition of 1 mM MgCl2 and quick cooling on ice. The concentration of the tRNA was determined by measuring the absorbance at 260 nm using a calculated extinction coefficient of 87,500 L·mol⁻¹·cm⁻¹ (43). Plateau values for fully charged tRNALeu transcript indicated that ~55% of the tRNA pool was competent for leucylation by yeast mitochondrial LeuRS.

Post-transfer Editing Assays—Yeast mitochondrial tRNALeu was aminoacylated with [3H]isoleucine (92 Ci/mmol) by an edit-

2 J. L. Hsu and S. A. Martinis, unpublished data.
ing-defective *E. coli* LeuRS mutant at 1 μM in a reaction containing 60 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 4 mM ATP, and 8 μM yeast mitochondrial tRNA⁰⁰⁰. The reaction was incubated for 3 h at room temperature and quenched with 0.18% acetic acid. Protein was removed by extraction with 1 volume of phenol: chloroform:isoamyl alcohol (125:24:1), pH 5.2 (Fisher Biotech, Fair Lawn, NJ). A half-volume of 4.6 M ammonium acetate, pH 5.0, was added, and the mischarged tRNA was ethanol-precipitated. The dried pellet was resuspended in 50 mM KPi buffer, pH 5.0. A total of 140 pmol of mischarged tRNA was obtained.

Hydrolysis assays were carried out using 60 mM Tris, pH 7.5, 10 mM MgCl₂, 150 mM KCl, and 300 mM mischarged yeast mitochondrial tRNA⁰⁰⁰. As indicated above, including KCl increases the post-transfer editing activity of yeast mitochondrial LeuRS.² The reaction was initiated by the addition of 50 nM enzyme and carried out at room temperature (15). At specific time points, 5 μl of the reaction were quenched on filter pads that were presoaked in 5% trichloroacetic acid and then washed and dried as described above.

**Yeast Complementation Assays**—The yeast null strain HM402 (MATα ade2-1 his3-11,15 leu2-3, 112 trp1-1 ura3-1 can1-100 nam2Δ::LEU12 (5 introns)) carrying an allelic disruption in the nam2 gene encoding yeast mitochondrial LeuRS (44, 45) and expressing a mitochondrial LeuRS via a maintenance plasmid YEpGMC063 (URA3⁰) was used for complementation assays (40). The HM402 strain contains an ade2 deletion, which enables the cells to develop a red pigment when the mitochondria are functional.

Strains were transformed with plasmids encoding wild-type (ymLEURST) or TT/VV (pVK042), R265A (pVK100) or D357A (pVK102) editing-defective mutant yeast mitochondrial LeuRSs, wild-type (pKIRAN) or TT/VV (pVK105), R249A (pVK106) or D345A (pVK108) *E. coli* LeuRSs (TRP1 marker), as well as an empty vector (pQB153T) for a negative control. The upstream region of each *E. coli* LeuRS gene encoded a mitochondrial target sequence to allow import of the heterologous protein into the yeast mitochondria. Transformants were selected on synthetic complete medium containing 2% glucose and lacking leucine, tryptophan, and uracil (40). Subsequently, transformants were grown on 5-fluoroorotic acid plates, as well as on antibiotic plates. Overnight cultures were transferred to 50 ml of MS-Amp/Kan/Str medium. A 500-μl aliquot of the suspension was spotted on MS-Amp/Kan/Str plates. An aliquot of 100 μl of 100 mM isoleucine was incorporated into a bored out well in the center of the plate. The isoleucine diffused to generate a concentration gradient of isoleucine around the well. Plates were incubated at 30 or 42 °C for 48–72 h. The diameter of the halo was measured, and the diameter of the well (1 cm) was subtracted from the halo diameter.

**Cell Growth Curves**—The yeast null strain HM402 harboring either the wild-type or editing-defective D357A mutant yeast mitochondrial LeuRS was grown in 100 ml of synthetic complete Leu⁻ Trp⁻ medium containing 2% glycerol, 0.1% fluoroorotic acid in the presence or absence of 50 μM excess isoleucine. Cells were grown at 30 °C with constant shaking at 200 revolutions/min for a period of 48 h. Absorbance was measured at specific time points at 600 nm.

For *E. coli* growth curves, 3-ml cultures of *E. coli* KL231 harboring either wild-type or the editing-defective D345A mutant *E. coli* LeuRS were grown at 30 °C in MS-Amp/Kan/Str medium. Cells were induced as described above to allow the expression of the enzymes. The cells were transferred to 100 ml MS-Amp/Kan/Str medium that either lacked or contained 10 mM excess isoleucine. The cells were grown at 200 revolutions/min for a period of 48 h at 42 °C. Absorbance measurements were taken at specific time points at 600 nm.

**RESULTS**

**Yeast Mitochondrial LeuRS Is Active in Post-transfer Editing**—The CPA domain of LeuRS contains a threonine-rich region that comprises a portion of the editing active site (13, 15, 31, 33, 46). A second downstream region is marked by a universally conserved aspartic acid that forms a hydrogen bond with the amino moiety of the bound amino acid (Fig. 1) (13). These regions are highly conserved in LeuRSs and also shared with the homologous IleRS and ValRS editing domains. Mutational analysis within these two regions has identified sites that alter or abolish amino acid editing activity of these synthetases (13, 15, 32, 34, 36).

We introduced mutations into yeast mitochondrial LeuRS, which had been shown to decrease or eliminate editing activity of LeuRSs from other origins. This included a double mutation of two neighboring threonines (Thr-263 and -264) to valine (TT/VV), which abolishes post-transfer editing activity of *E. coli* LeuRS (34). The universally conserved aspartic acid (Asp-357) of yeast mitochondrial LeuRS was also mutated to

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³ The abbreviation used is: MS, minimal standard.
alanine and was expected to eliminate amino acid editing activity similar to analogous mutations in IleRS, ValRS, and other LeuRSs (13, 35, 36). We also introduced an alanine for the conserved arginine 265 (R265A), which had minimal effects on post-transfer editing activity for E. coli LeuRS (15).

The wild-type and mutant yeast mitochondrial LeuRSs were purified to homogeneity via affinity chromatography using a His$_6$ tag. All purified enzymes exhibited similar leucylation activities that were comparable with the wild-type enzyme (Fig. 2A). The specific activities for the mutant enzymes were similar to the wild-type LeuRS (17,684 units/mg protein). Similar to E. coli LeuRS, the TT/VV yeast mitochondrial LeuRS mutant had substantially decreased post-transfer editing activity and yielded mischarged Ile-tRNALeu. Likewise (as found for the same mutation in other LeuRS (13), IleRS (35), and ValRS (36) enzymes), the D357A mutant LeuRS from yeast mitochondria abolished editing activity and misaminoacylated isoleucine to tRNALeu. Interestingly, the R265A mutation in yeast mitochondrial LeuRS also abolished post-transfer editing activity (Fig. 2B), even though the homologous mutation in E. coli LeuRS maintained wild-type-like editing activity (15). Mischarging assays showed that the R265A LeuRS also produced Ile-tRNALeu (Fig. 2C).

Editing-defective LeuRSs Are Tolerated by Yeast Mitochondria—We tested each of the yeast mitochondrial editing-defective LeuRS enzymes in complementation assays to screen for effects on cell growth and survival. The complementation assay uses a yeast null strain (HM402) that has a genomic disruption of the gene that encodes mitochondrial LeuRS, which is replaced by insertion of a LEU2 marker (44, 45). To maintain this disruption, complementation assays are carried out under limiting leucine conditions. A maintenance plasmid with a LIRA3 marker expresses the essential mitochondrial LeuRS. Significantly, HM402 also lacks mitochondrial introns that require a secondary activity of yeast mitochondrial LeuRS, which aids in the splicing of two related group I introns (47, 48). Thus, complementation of HM402 is solely based on the effectiveness of the LeuRS enzyme in protein synthesis.

The yeast strain HM402 was transformed with plasmids expressing each of the yeast mitochondrial wild-type and edit-
We tested complemented yeast cells in solution cultures in attempts to identify more subtle changes in growth curves. The yeast null strain HM402 harboring wild-type or the D357A editing-defective LeuRS enzyme was grown over 48 h in minimal medium that completely lacked leucine but contained 50 mM isoleucine. The growth curve overlapped between the wild-type and D357A mutant LeuRS-complemented cells, whether excess isoleucine was present or absent (Fig. 3B). Thus, despite the fact that the yeast mitochondrial LeuRS has a viable and robust post-transfer editing activity, it does not appear to be essential to the yeast mitochondria.

LeuRS Editing Defects Are Lethal to E. coli—We employed an E. coli temperature-sensitive strain (KL231) for complementation assays (37) that expresses a mutant LeuRS, which is inactive at 42 °C. E. coli KL231 was complemented by wild-type E. coli LeuRS (Fig. 4). However, the yeast mitochondrial enzyme failed to rescue the temperature-sensitive phenotype (Fig. 4B), even though the yeast enzyme recognizes E. coli tRNA_Leu as a substrate in vitro (49). It is possible that the yeast mitochondrial LeuRS is simply not very stable at 42 °C and hence failed to complement the temperature-sensitive strain. Indeed, attempts to express yeast mitochondrial LeuRS at 37 °C in E. coli BL21 cells for in vitro studies significantly reduces protein production.

We transformed E. coli KL231 with plasmids expressing the editing-deficient D345A and TT/VV E. coli LeuRS mutants. We also included an R249A mutant LeuRS that had minimal, if any, effect on the editing of E. coli LeuRS. Each of the mutant E. coli LeuRSs complemented the temperature-sensitive strain at 42 °C. However, when high concentrations of 100 mM isoleucine were introduced into a central well on the agar plates, E. coli cells that were dependent on an editing-defective LeuRS yielded a halo or zone of growth inhibition (Fig. 5A). This is consistent with previous complementation experiments (where a-aminobutyrate was toxic to E. coli) that were dependent on a T252D or T252E LeuRS mutation (27). The editing deficiency is toxic to E. coli in the presence of excess isoleucine. This contrasts with yeast, where editing-defective mutants failed to alter complementation activity. Interestingly, the R249A (Arg-265 of yeast mitochondrial LeuRS) mutation also differentially affected the editing activities of E. coli LeuRS and yeast mitochondrial LeuRS in vitro. The R249A mutation of E. coli LeuRS has very little, if any, mis-charging activity in vitro (15). This is consistent with the lack of any halo in the complementation assays that relied on E. coli R249A LeuRS (Fig. 5A).

Despite the presence of 100 mM isoleucine in the central well, no halo was observed for cells grown at 30 °C, suggesting that the endogenous wild-type LeuRS is sufficiently supplying correctly charged tRNA\textsuperscript{Leu}, even when editing-defective LeuRSs are present. In addition, as would be expected, controls in which leucine was incorporated into the central well did not affect the growth of E. coli KL231 when complemented by wild-type or editing-deficient LeuRSs (data not shown).

We also measured the growth rates of E. coli KL231 harboring either the wild-type or editing-defective D345A mutant of E. coli LeuRS in liquid minimal medium cultures. As shown in Fig. 6A, the growth curves for the wild-type and D345A-com-

![FIGURE 3. Yeast mitochondrial complementation activity of wild-type and editing-defective LeuRS mutants.](Image)
implemented E. coli cells overlapped at 42 °C. However, in the presence of 10 mM isoleucine and 0.8 mM leucine, growth of the strain expressing the editing-defective mutant was reduced compared with the strain expressing the wild-type E. coli LeuRS. Transformants were streaked on MS medium including 0.8 mM isoleucine and leucine and the following antibiotics: kanamycin (30 μg/ml), streptomycin (50 μg/ml), and ampicillin (100 μg/ml) (MS-Amp/Kan/Str) and grown at 30 °C (A) or 42 °C (B).

**FIGURE 5.** Isoleucine sensitivity of E. coli KL231 complementation activity for wild-type and editing-defective E. coli LeuRS. A, halo assay for E. coli strain KL231 was carried out by transformation with plasmids expressing wild-type (WT; p15sec3-1) or TT/VV (pYZHA13) and R249A (pMURe6) or D345A (pHAPPY2-1-1-28) mutant E. coli LeuRS enzymes. Zones of inhibition or halos are marked by circles around a central well bored into the agar that contains 100 mM isoleucine. B, histogram representing the halo diameter in response to isoleucine toxicity for amino acid editing-defective E. coli LeuRS mutants.

LeuRS Editing Defects Do Not Alter Mitochondrial Viability—Because E. coli LeuRS editing-defective enzyme mutants were toxic to E. coli, we tested them for their effects on the yeast mitochondria. E. coli LeuRS was fused to a mitochondrial targeting sequence as described previously (40). The yeast null mutant HM402 was complemented by the E. coli LeuRS gene that expressed this fusion protein (Fig. 7). As found for the yeast mitochondrial mutants that were defective in post-transfer editing, the E. coli LeuRS editing defects also failed to decrease the viability of yeast mitochondria.

We hypothesized that the yeast mitochondria may have evolved to tolerate higher levels of infidelities in protein synthesis. Alternately, this organelle may manage fidelity via a different mechanism. The human mitochondrial LeuRS, which com-
pletely lacks a viable post-transfer editing mechanism (50), has evolved higher discrimination of leucine versus isoleucine because of a differential in the $K_m$. We measured a $K_m$ of 170 $\mu$M for leucine activation by yeast mitochondrial LeuRS, which is similar to the human enzyme (130 $\mu$M (50)). Both are significantly higher than the $K_m$ of $\sim 10$ $\mu$M for E. coli LeuRS (23, 50). Likewise, the $K_m$ value for isoleucine activation by yeast mitochondrial LeuRS was measured to be 10.5 mM, which compares to the human mitochondrial LeuRS value of 14 mM (50). The yeast mitochondrial LeuRS $k_{cat}$ parameters for leucine ($3.3 \text{ s}^{-1}$) was similar to previously measured values (50) for E. coli and human mitochondrial LeuRS, but the $k_{cat}$ for isoleucine ($0.94 \text{ s}^{-1}$) was slightly increased. Unlike the human enzyme, the yeast mitochondrial LeuRS has maintained a viable post-transfer editing active site, but its activity is likely far less critical compared with E. coli LeuRS.

DISCUSSION

LeuRSs carry out amino acid editing to clear mistakes that can include a number of different amino acids. As would be expected, yeast mitochondrial LeuRS has also maintained an active site for post-transfer editing of mischarged tRNA. Similar to E. coli LeuRS (13, 34), simultaneous mutations of the conserved threonines or aspartates abolished the post-transfer editing function of the enzyme and allowed mischarging of isoleucine to tRNA$^{Leu}$. Surprisingly, in vivo analysis suggests that defects in the editing active site that might result in errors in protein synthesis do not appear to alter the viability of the yeast mitochondria. This contrasts with E. coli, where complementation assays showed that editing defects were toxic.

It is possible that the mitochondria is tolerant of increased infidelities during translation or has other mechanisms, which can suppress or dampen these mistakes. Interestingly, human mitochondrial LeuRS has a defunct editing active site in its CP1 domain and is not even capable of amino acid editing (50). This editing-defunct LeuRS also complements yeast mitochondrial null strains (40). The human mitochondrial enzyme has optimized fidelity by evolving higher affinity and specificity for cognate leucine as compared with isoleucine. A comparison of the $K_m$ for leucine and isoleucine suggests that the yeast mitochondrial LeuRS operates via a similar strategy for the discrimination of leucine and isoleucine. Thus, unlike the human enzyme, the yeast mitochondrial LeuRS has clearly maintained a competent editing active site that deacylates mischarged tRNA in vitro. Nevertheless, under physiological conditions, the yeast mitochondrial enzyme may not require editing activity to maintain fidelity.

Although there are no direct reports of measurements for mitochondrial concentrations of leucine and isoleucine that we could identify, the total yeast concentrations for leucine and isoleucine range from 0.45 to 1.5 mM depending on growth conditions.
Editing of Yeast Mitochondrial Leucyl-tRNA Synthetase

It has been suggested that leucine is actively transported into the mitochondria (52, 53) but under tight regulation that depends on its requirement for economical consumption during mitochondrial protein synthesis (53). The mechanism of transport for isoleucine is less clear, but it has been estimated that isoleucine and leucine are present at comparable intracellular levels (54). Thus, even though the yeast mitochondrial LeuRS maintains an active amino acid editing mechanism, it is possible that fidelity is maintained by carefully controlling the mitochondrial concentrations of amino acids.

Interestingly, other mitochondrial aminoacyl-tRNA synthetases with counterparts that exhibit amino acid editing have been characterized that also lack editing functions. For example, yeast mitochondrial phenylalanyl-tRNA synthetase (PheRS) does not exhibit an amino acid editing activity. The wild-type PheRS even generates Tyr-tRNA_Phe in vitro (55). Human prolyl-tRNA synthetase (ProRS) is also missing an editing function and fails to clear mischarged tRNA_Phe (56). It is possible in these cases or even the human mitochondrial LeuRS case that an independent editing domain, such as the YbaK protein (57), clears mischarged products. However, these factors that edit in trans have not yet been identified in yeast mitochondria. Moreover, unlike these mitochondrial enzymes that lack editing activities, the yeast mitochondrial LeuRS appears to maintain a robust post-transfer editing activity.

Notably, the yeast mitochondrial LeuRS is also required for excising two closely related introns called bI4 and aI4. These respectively interrupt the genes for cytochrome b and the α-subunit of cytochrome oxidase, which are both essential respiratory proteins (47, 48). It is clear that the CP1 editing domain is important to this RNA splicing activity (58). Fragments of the isolated yeast mitochondrial LeuRS CP1 domain bind and confer splicing of the bI4 intron (58). The molecular role of the CP1 editing domain in RNA splicing is not clear. Specific sites that affect RNA splicing have been mapped to the CP1 domain (58) but not to the post-transfer editing active site. If aspects of a competent post-transfer editing active site contribute to RNA splicing, then the molecular landscape of this hydrolytic active site may have been maintained for a secondary but essential role, namely to excise group I introns from critical respiratory proteins. It is also possible that the yeast mitochondrial LeuRS has evolved to effectively use its CP1 domain for the essential splicing function, because its amino acid editing activity appears to be less critical under the physiological conditions of the mitochondria.

The vast majority of proteins involved in the mitochondrial function are encoded by the nuclear genome and imported into this subcellular compartment (59). Significantly, mitochondrial protein synthesis is responsible for synthesizing just thirteen mitochondrial proteins from the condensed mitochondrial genome (60). By virtue of the very small number of proteins that are synthesized in the mitochondria, this limited set of mitochondrial proteins may also tolerate lower fidelity. A robust protein degradation mechanism in mitochondria (61) could also serve as the primary means to address reduced accuracy in protein synthesis by clearing incorrectly folded protein. Thus, it is possible that the CP1 editing domain, which also functions as an RNA splicing domain in yeast mitochondrial LeuRS, may have been selectively optimized for a more critical role in excising introns from essential genes.

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