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Hervé Roy
The Ohio State University

Jiqiang Ling
The Ohio State University

Juan D. Alfonzo
The Ohio State University

Michael Ibba
Chapman University, ibba@chapman.edu

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Loss of Editing Activity during the Evolution of Mitochondrial Phenylalanyl-tRNA Synthetase*

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Hervé Roy[‡], **Jiqiang Ling**[§], **Juan Alfonzo**[‡§], **and Michael Ibba**[‡§1]

From the [‡]*Department of Microbiology and the* [§]*Biochemistry Program, The Ohio State University, Columbus, Ohio 43210-1292*

Accurate selection of amino acids is essential for faithful translation of the genetic code. Errors during amino acid selection are usually corrected by the editing activity of aminoacyl-tRNA synthetases such as phenylalanyl-tRNA synthetases (PheRS), which edit misactivated tyrosine. Comparison of cytosolic and mitochondrial PheRS from the yeast *Saccharomyces cerevisiae* suggested that the organellar protein might lack the editing activity. Yeast cytosolic PheRS was found to contain an editing site, which upon disruption abolished both *cis* and *trans* editing of Tyr-tRNA^{Phe}. Wild-type mitochondrial PheRS lacked *cis* and *trans* editing and could synthesize Tyr-tRNA^{Phe}, an activity enhanced in active site variants with improved tyrosine recognition. Possible *trans* editing was investigated in isolated mitochondrial extracts, but no such activity was detected. These data indicate that the mitochondrial protein synthesis machinery lacks the tyrosine proofreading activity characteristic of cytosolic translation. This difference between the mitochondria and the cytosol suggests that either organellar protein synthesis quality control is focused on another step or that translation in this compartment is inherently less accurate than in the cytosol.

The aminoacyl-tRNA synthetases (aaRS)[2] are a ubiquitous and essential protein family required for protein synthesis (1–3). Structurally and functionally the aaRSs are divided into two unrelated but biochemically analogous groups, class I and class II (4–6). The aaRSs attach amino acids to the 3′-ends of tRNA containing the corresponding anticodon sequence, and the resulting aminoacyl-tRNAs (aa-tRNAs) are used as substrates for ribosomal translation of mRNA. The accuracy of aa-tRNA synthesis is generally assured by the existence of aaRSs specific for each particular amino acid-tRNA pair. Cognate tRNA recognition and discrimination of non-cognate RNAs are achieved by sequence-specific direct and indirect readout of the numerous combinations of bases present in tRNAs (7–10). The relative structural simplicity and inherent similarity between the amino acid substrates makes their accurate recognition and discrimination more challenging. Although some amino acids such as cysteine and tyrosine are different enough to allow their specific recognition by a particular aaRS (11, 12), others such as valine and isoleucine are less easily distinguished. For example the class I aaRS isoleucyl-tRNA synthetase (IleRS) is only able to poorly discriminate against valine, which has a misactivation rate of about 1:200 compared with the cognate substrate isoleucine. Despite this significant rate of misactivation and misaminoacylation, the accuracy of translation is not compromised because of the existence of an intrinsic proofreading and editing mechanism in IleRS that specifically hydrolyzes both misactivated Val-AMP and misaminoacylated Val-tRNA^{Ile} (13, 14). In addition to IleRS, many other class I and class II aaRSs also employ editing to prevent release of non-cognate aa-tRNA and subsequent loss of translational accuracy (reviewed in Refs. 15 and 16). With a few notable exceptions (17), editing generally occurs in specialized domains distal from the active site such as the class I-specific CP1 region of IleRS and leucyl-, and valyl-tRNA synthetases. The editing domains of class II aaRSs are more diverse than their class I counterparts and include the "H*XXX*H" domain found in both alanyl- (18) and threonyl-tRNA synthetases (ThrRS) (19), an unrelated domain in archaeal ThrRS (20, 21), the Ybak-like domain in prolyl-tRNA synthetase (ProRS) (22–24), and the B3/B4 domain of phenylalanyl-tRNA synthetase (PheRS) (25).

The editing domains of aaRSs are normally found in the same subunit as the active site, the only known exceptions being PheRS and certain examples of ProRS and ThrRS. PheRS is normally an $(\alpha\beta)_2$ heterotetramer, with the active site located in the α-subunit and tRNA binding sites in both subunits. Recent studies in bacteria revealed that hydrolysis of misaminoacylated Tyr-tRNA^{Phe} occurs at an editing site in the β-subunit ~40 Å from the active site (25). This editing site, at the boundary of the B3 and B4 subdomains, is highly conserved in bacterial PheRSs and aligns with a divergent domain conserved in eukaryotic and archaeal sequences that is believed to participate in proofreading. It is less clear whether mitochondrial PheRSs also have the potential to edit misacylated tRNAs, as they are monomers and thus lack the conventional $(\alpha\beta)_2$ oligomeric form (26, 27). Mitochondrial PheRS sequences are most closely related to the bacterial type, being chimeras of the α-subunit (with an insertion between motifs 2 and 3) and the C-terminal tRNA anticodon binding domain (B8) of the β-subunit (26). Despite their similarity to bacterial PheRSs, the mitochondrial versions do not contain regions analogous to the known editing domain (25). Although it was originally suggested that mitochondrial PheRSs were active in editing (28), later studies questioned the purity of the enzymes used for these studies (26). Here we describe an investigation of amino acid specificity and editing by mitochondrial and cytosolic PheRSs from the yeast *Saccharomyces cerevisiae*. Both enzymes display a lack of specificity toward Phe and are able to bind Tyr. The cytosolic enzyme is shown to be less specific than its mitochondrial counterpart but contains an editing site in the β-subunit that specifically edits misacylated Tyr-tRNA^{Phe}. The mitochondrial enzyme is deprived of such an activity and is also able to synthesize the misaminoacylated species Tyr-tRNA^{Phe}. Editing activity toward Tyr-tRNA^{Phe} is also absent from mitochondrial extracts, indicating the lack of this quality control step *in vivo*. These findings are discussed in the context of other recent studies, which together with the data presented here, raise the possibility that mitochondrial protein synthesis may be less accurate than its cytosolic counterpart.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and General Methods—Plasmids carrying cytosolic or mitochondrial yeast tRNA^{Phe} genes for T7 runoff transcription

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[1] To whom correspondence should be addressed: Dept. of Microbiology, The Ohio State University, 484 W. 12th Ave., Columbus, OH 43210. Tel.: 614-292-2120; Fax: 614-292-8120; E-mail: ibba.1@osu.edu.

[2] The abbreviations used are: aa, aminoacyl; RS, tRNA synthetase.

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were gifts from O. Uhlenbeck (Northwestern University). The strains *Escherichia coli* XL1-Blue/pQE31-FRS-ec (producing His_6-tagged *E. coli* PheRS) and *E. coli* BL21/pQE32-FRS-sc (producing His_6-tagged cytosolic *S. cerevisiae* PheRS) were gifts from D. A. Tirrell (California Institute of Technology). *E. coli* BL21-RIL/pET16b producing His_6-tagged mitochondrial *S. cerevisiae* PheRS was a gift from R. A. Zimmermann (University of Massachusetts). All His_6-tagged proteins were purified to >99% purity on nickel-nitrilotriacetic acid-agarose (Qiagen) by standard procedures. The specific activities of the purified proteins (nmol of Phe attached/mg/min) were: wild-type cytoplasmic PheRS, 22; D243A cytoplasmic PheRS, 12; wild-type mitochondrial PheRS, 89; and A333G mitochondrial PheRS, 73. Point mutations were introduced into the *pheST* genes (encoding the PheRS α- and β-subunits, respectively) by PCR with two self-complementary 33-mer oligonucleotides that carried the appropriate mutations. Reactions were performed with the QuikChange site-directed mutagenesis kit (Stratagene). Introduction of the desired mutations was monitored by sequencing of the resulting genes. Commercial L-Tyr (Sigma) was shown to be free of phenylalanine (Phe) contamination by pyrophosphate exchange before and after recrystallization of the amino acid as described previously (29). LB and M9 media were prepared as described previously (30). All buffers were adjusted to the correct pH with NaOH unless otherwise indicated. Immunoblotting was performed as described previously (31) using rabbit polyclonal antibodies raised against yeast cytosolic PheRS (AnimalPharm, Healdsburg, CA). $tRNA^{Trp}$ and tryptophanyl-tRNA synthetase were prepared as described previously (31).

Preparation of in Vitro Transcribed $tRNA^{Phe}$—In vitro T7 RNA polymerase runoff transcription reactions were conducted according to standard procedures (32). After ethanol precipitation, tRNA transcripts were resuspended by heating in 10 mM Hepes (pH 7.2), 1 mM EDTA, and 7 M urea, loaded on a Resource Q 6 anion exchange column (Amersham Biosciences) and eluted with a gradient of 0–1 M NaCl in the loading buffer. Fractions containing tRNA were pooled and desalted on a PD-10 column (Amersham Biosciences) against 10 mM Hepes (pH 7.2). The transcripts were ethanol precipitated, washed with 80% ethanol, dried, resuspended in 10 mM Hepes, pH 7.2, and 2 mM $MgCl_2$, and finally refolded by incubation for 1 min at 75 °C followed by slow cooling down to room temperature.

ATP-PPi Exchange Reaction—The reaction was carried out at 37 °C in a medium containing 100 mM Na-Hepes (pH 7.2), 30 mM KCl, 10 mM $MgCl_2$, 2 mM NaF, 2 mM ATP, 2 mM [^{32}P]PPi (1 cpm/pmol), various amounts of Phe (1.5–300 μM) and Tyr (0.2 to 7 mM), and 13 nM cytosolic PheRS or 10–70 nM mitochondrial enzyme. After 1–5 min, 25 μl of the reaction were removed and added to a solution containing 1% charcoal, 5.6% $HClO_4$, and 75 mM PPi. The radiolabeled ATP bound to the charcoal was filtered through a 3MM Whatman filter disc under vacuum and washed three times with 5 ml of water and once with 5 ml of ethanol. The filters were dried, and the radioactivity was counted by liquid scintillation counting (Ultima Gold, Packard Instrument Co.).

Aminoacylation Assay with Radiolabeled Amino Acids—Aminoacylation was performed in 100 mM Na-Hepes (pH 7.2), 30 mM KCl, 2 mM ATP, 10 mM $MgCl_2$, 25 μM L-[^{14}C]Phe (280 cpm/pmol), 5 μM tRNA transcript, and 10–100 nM yeast PheRS. 15-μl aliquots were spotted on 3MM filter disks (Whatman), washed three times in 10% trichloroacetic acid, and dried. The amount of radioactivity retained was determined by liquid scintillation counting. One unit of PheRS corresponded to the amount of enzyme necessary to catalyze the formation of 1 nmol of Phe-$tRNA^{Phe}$ min^{-1} mg^{-1} protein at 37 °C.

Aminoacylation Assay with Radiolabeled tRNA—Synthesis of tRNA ^{32}P-labeled pA76 transcripts were performed essentially as described previously (33) except that the CCA-3′-end was removed prior to labeling. Briefly, the CCA-3′-terminal nucleotides of the tRNA were first removed by treatment of 20 μM tRNA transcript with 73 μg/ml *Crotalus atrox* venom (Sigma) in a buffer containing 40 mM sodium Gly (pH 9.0) and 10 mM magnesium acetate. The mix was incubated for 2 h at 21 °C and phenol/chloroform extracted and ethanol precipitated and finally desalted by gel filtration through a Sephadex G 25 column (Amersham Biosciences). The CCA-3′-end of the tRNA was reconstituted and radiolabeled by incubation for 10 min at 37 °C with 0.5 μM snake venom-treated tRNA in 50 mM Na-Gly (pH 9.0), 10 mM $MgCl_2$, 10 μM CTP, 9 μM ATP, 1 μM [α-^{32}P]ATP with 3 μg/ml *E. coli* tRNA-terminal nucleotidyltransferase (34) in a final volume of 20 μl. The reaction was stopped by the addition of 1 volume of phenol, and the resulting mixture was gel filtered twice through a G25 column. As described previously (33), the aminoacylation reaction was performed in a 10-μl aminoacylation medium (see above) containing 5 mM cold amino acids or Phe analogues, 5 μM transcript, and a trace of radiolabeled tRNA. After 15 min of incubation an aliquot was removed and incubated for 30 min at room temperature with P1 RNase. The liberated [α-^{32}P]AMP and aminoacyl-[α-^{32}P]AMP were separated by TLC on polyethyleneimine cellulose and visualized as described previously (33).

Post-transfer Editing Assay—The cytosolic or mitochondrial Tyr-$tRNA^{Phe}$ and Phe-$tRNA^{Phe}$ (*S. cerevisiae* transcripts) were prepared as described previously (25) in an aminoacylation reaction containing 30 μM [^{3}H]Tyr (180 cpm/pmol), 0.5 μM mitochondrial PheRS A333G, and 5 μM corresponding *in vitro* transcribed $tRNA^{Phe}$. Comparison to the total tRNA concentration then allowed us to estimate that purified Phe-$tRNA^{Phe}$ yields were about 20% (*i.e.* these preparations also contained 80% uncharged tRNA), whereas Tyr-$tRNA^{Phe}$ yields were 15% (85% uncharged), within the typical range expected for aminoacyl-tRNA preparations. Post-transfer editing reaction mixtures contained 100 mM Na-Hepes (pH 7.2), 30 mM KCl, 10 mM $MgCl_2$, 1 μM [^{3}H]Tyr-$tRNA^{Phe}$, and a catalytic amount of PheRS (5 nM cytosolic or 5 nM to 2 μM mitochondrial yeast PheRS). The mixture was incubated at 37 °C, and the post-transfer editing reaction was followed by measuring the remaining radiolabeled aa-tRNA in aliquots of 15 μl after 0–12 min of incubation as described for the aminoacylation assay (see above). The amount of cytosolic or mitochondrial crude extract from *S. cerevisiae* used in the editing assay was determined according to the PheRS specific activity measured for the extracts, 0.78 nmol/min/mg of protein and 0.19 nmol/min/mg of protein, respectively.

Preparation of Cytosolic and Mitochondrial Fractions of S. cerevisiae—*S. cerevisiae* strain W303 was grown in rich medium containing 2% galactose, and mitochondria were isolated as described previously (35). Spheroplasts were prepared by Zymolyase-20T (ICN) treatment and broken by three passes through an EmulsiFlex®-C5 (AVESTIN) in 0.6 M sorbitol (Fluka), 10 mM Tris-HCl (pH 7.4), and 1 mM phenylmethylsulfonyl fluoride. Mitochondria were purified by centrifugation and extensive washing in the same buffer as described previously (36). The cytosolic fraction obtained during this procedure was clarified by ultracentrifugation at 100,000 × *g*. Highly purified organelles were obtained by centrifugation at 100,000 × *g* (Beckman SW41) in a Percoll™ step gradient (Amersham Biosciences) (40% Percoll™ in 0.6 M sorbitol (Fluka), 10 mM Tris-HCl (pH 7.4), overlaid with 20% Percoll™ in the same buffer). These procedures yielded cytosolic and mitochondrial fractions with less than 0.5% cross-contamination as judged by Western blot analysis with antibodies specific for proteins in the

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different compartments as described elsewhere (37). Mitochondria were suspended in aminoacylation buffer (see above) followed by sonication for 1 min at 50% output with a Sonifier 450 (Branson) equipped with a microprobe. The resulting extract was centrifuged at 100,000 × *g* for 1 h (S100) to precipitate the membrane fractions. The resulting soluble extracts were used for aminoacylation assays.

Phylogenetic Analyses—179 PheRS α-subunit sequences (92 from eubacteria, 23 from archaea, and 35 and 29 cytosolic and mitochondrial eukaryotic sequences, respectively) were aligned with the ClustalX program version 1.83 (38). The alignment process was guided with the known three-dimensional structure of PheRS of *Thermus thermophilus* and refined manually. The trees were generated by applying the neighbor-joining method with the program ClustalX or the Phylip package version 6.63 (39) to a set of 100 bootstrap replicates of the ungapped alignment. Maximum likelihood analysis was also applied to the same alignment with the program Puzzle version 5.2 (40) where more than 50×10^6 quartets were analyzed. The trees were rooted using the nine known sequences of *o*-phosphoseryl-tRNA synthetase found in methanogenic archaea that were previously identified as paralogues of the α-subunit of PheRS (41).

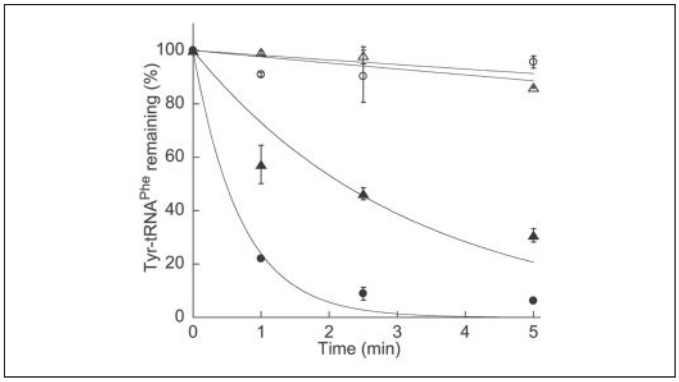

FIGURE 1. **Specific deacylation of Tyr-tRNA^{Phe} by yeast cytosolic PheRS.** Reactions were performed with 1 μM Tyr-tRNA^{Phe} prepared from *in vitro* transcribed yeast cytosolic tRNA^{Phe} (*circles*) or yeast mitochondrial tRNA^{Phe} (*triangles*) with the addition of 5 nM cytosolic PheRS (*filled symbols*) or in the absence of enzyme (*open symbols*). Values shown are the means of three independent experiments, with *error bars* representing ± 1 S.D. The possible contribution of nonspecific nuclease activity to the observed deacylation was excluded by the finding that cytosolic PheRS did not significantly deacylate Phe-tRNA^{Phe} after its synthesis (see for example Fig. 3*C*).

RESULTS

The β-Subunit of Cytosolic PheRS Contains a Post-transfer Editing Site—Previous studies (42, 43) suggested that yeast cytosolic PheRS could hydrolyze Tyr-tRNA^{Phe}, as recently directly demonstrated for the *E. coli* enzyme (25). Tyr-tRNA^{Phe} was synthesized and added to a deacylation reaction containing yeast cytosolic PheRS. Cytosolic PheRS was able to specifically deacylate Tyr-tRNA^{Phe} derived from both cytosolic and mitochondrial tRNAs, indicating the presence of *trans* editing activity (Fig. 1). Amino acid sequences from 179 PheRSs were aligned to investigate whether this activity in the yeast enzyme could be attributed to a catalytic site in the β-subunit, as is the case in *E. coli*. Examination of the PheRS β-subunit sequence alignment allowed identification of domains B1, B3, and B4 (B2 is absent from eukaryotes), but B3/B4 residues which are involved in bacterial editing were not well conserved in their eukaryotic counterparts (Fig. 2). The average percentage amino acid identities observed within eubacterial and eukaryotic B3/B4 ungapped domain alignments were 42 and 57%, respectively, whereas there was far less similarity when eukaryotic and eubacterial B3/B4 domains were compared with each other (15% identity). No conservation was seen at the small residues Thr^{354} and Ala^{356}, changes in which ablated editing by *E. coli* PheRS. Moderate conservation was observed at His^{265} and Glu^{334} (His^{158} and Asp^{243}, respectively, in yeast PheRS) suggesting that those residues might contribute to the editing activity of the cytosolic enzyme. Based on this analysis, cytosolic PheRS variants were produced containing the replacements βH158A and βD243A. Although the βH158A replacement had no effect (data not shown), introduction of βD243A led to a loss in Tyr-tRNA^{Phe} deacylation activity (Fig. 3*A*) similar to that observed for *E. coli* variants defective in editing (25). The loss of editing but not synthetic activity resulting from the βD243A replacement was confirmed by the ability of this PheRS variant to synthesize Tyr-tRNA^{Phe} (Fig. 3*B*). The misaminoacylation activity of PheRS βD243A was specific for yeast cytosolic tRNA^{Phe}, as mitochondrial tRNA^{Phe} was not a substrate for tyrosylation. This confirms previous indications that mitochondrial tRNA^{Phe} could not be phenylalanylated by cytosolic PheRS (Fig. 3*C*). This discrepancy between the aminoacylation and editing capacities of mitochondrial tRNA^{Phe}, together with the absence of an obvious editing domain, prompted us to investigate the organellar PheRS in more detail.

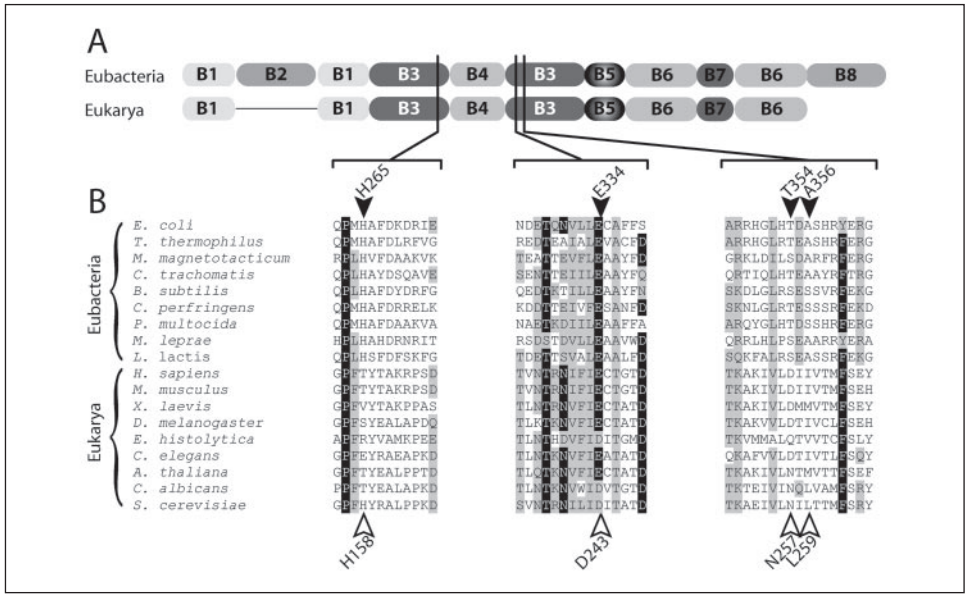

FIGURE 2. **Comparison of the β-subunits of eubacterial and eukaryotic PheRSs.** *A*, modular structure of the PheRS β-subunit. *B*, sampling of an alignment of the B3/B4 domain regions. Positions previously identified as being involved in the editing activity of *E. coli* PheRS are indicated with *black arrows*. The equivalent positions found in the yeast β-subunit are indicated with *white arrows*. The sequence alignment (44 eubacterial and 44 eukaryotic sequences) was *shaded* with the program Bioedit. *Gray boxes* indicate 60% conservation according to similarity matrix (PAM250)-based *shading*. *Black boxes* indicate residues identical in more than 60% of the sequences.

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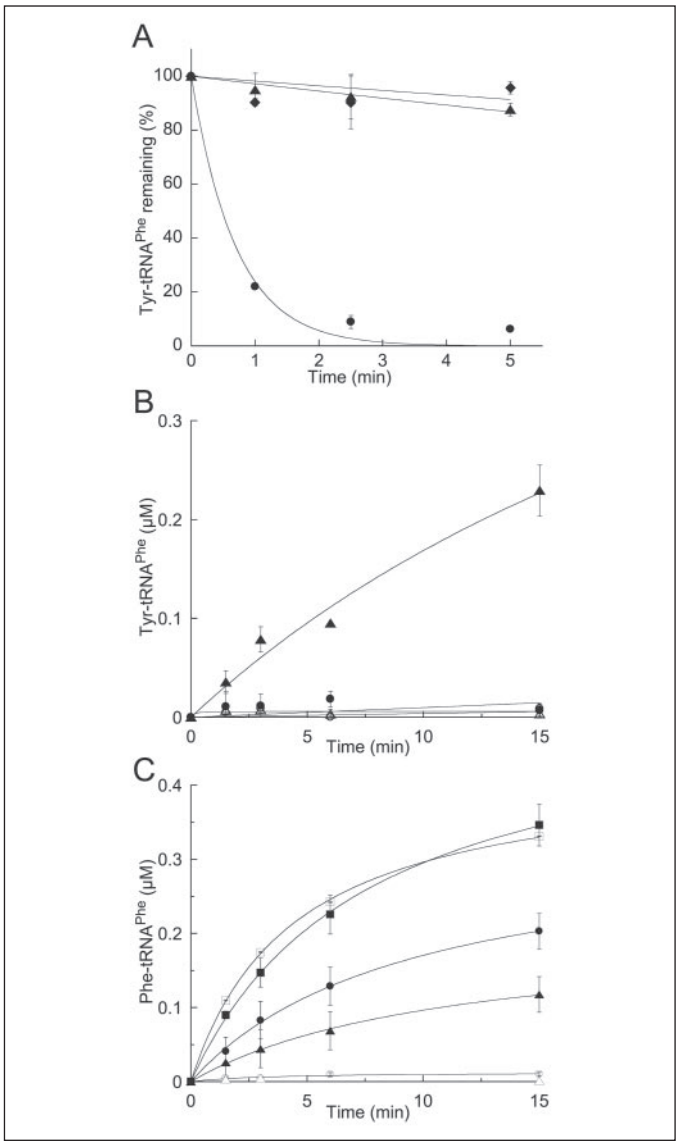

FIGURE 3. **Disruption of post-transfer editing by yeast cytosolic PheRS.** *A*, post-transfer editing of Tyr-tRNA^{Phe} by wild-type and variant PheRSs. Reactions were performed with 1 μM cytosolic Tyr-tRNA^{Phe} in the presence of 5 nM enzyme. *B*, tyrosylation of tRNA^{Phe} by cytosolic PheRS. Reactions were performed with 10 μM cytosolic tRNA or 3.5 μM mitochondrial tRNA and 0.1 μM PheRS. *C*, phenylalanylation of mitochondrial tRNA^{Phe}. Reactions were performed with 3.5 μM *in vitro* transcribed tRNA^{Phe} and 0.1 μM PheRS. *Circle*, wild-type cytosolic PheRS; *triangle*, editing-defective (*βD243A*) cytosolic PheRS; *square*, mitochondrial PheRS; *diamond*, without enzyme; *filled symbols*, cytosolic tRNA; *open symbols*, mitochondrial tRNA.

Selectivity of the Active Sites of Mitochondrial and Cytosolic PheRS—The specificity of the active sites of both yeast PheRSs toward Phe and Tyr was investigated by determining the catalytic constants for steady-state amino acid activation from ATP-PPi exchange kinetics (TABLE ONE). The mitochondrial PheRS was four times more efficient than the cytosolic PheRS with respect to Phe activation. This was mostly because of the 7-fold weaker apparent affinity of the cytosolic enzyme for its substrate compared with the mitochondrial enzyme. This tendency was reversed when Tyr was the substrate. Because Tyr exhibits poor solubility we were unable to saturate the mitochondrial enzyme. Instead, the k_{cat}/K_m was estimated at sub-saturating concentrations of Tyr. Although the cytosolic enzyme was twice as efficient for Tyr activation as its mitochondrial counterpart, for both enzymes the relative efficiency of activation of Phe was only 1–2 orders of magnitude higher than for Tyr. To determine whether such a poor apparent selectivity was because of the contamination of Tyr by a trace of Phe we assessed the purity of the Tyr substrate by mass spectrometry (data not shown). Even after several cycles of heating and cooling, the Tyr used for our experiments was found to be stable and free of any trace of Phe.

Specificity of Yeast Cytosolic PheRS Editing—To further define the specificity of the editing reaction several amino acids and Phe analogues were tested for their ability to be attached to tRNA^{Phe} by cytosolic PheRS and the editing-defective variant βD243A. The compounds used were the 20 canonical amino acids, three intermediates of Phe metabolism (phenylpyruvate, *p*-hydroxyphenylpyruvate, and prephenate), and four other Phe analogues (*p*-fluoro, *p*-chloro, *p*-bromo-, and *p*-amino-Phe) that are known to be charged but not edited by *E. coli* PheRS (25). In addition to Phe, *para*-halogenated Phe derivatives, *p*-amino-Phe, Leu, and Trp were attached to tRNA by both enzymes indicating that all were substrates for the synthetic site of the α-subunit but not for the editing site of the β-subunit (Fig. 4*A*). In the case of Trp, this supports previous data from *in vivo* misincorporation experiments (44). The βD243A variant also synthesized Tyr-tRNA^{Phe} indicating that this particular aminoacyl group is recognized by both the synthetic and editing sites of PheRS (Fig. 4*B*).

Mitochondrial PheRS Lacks the Capacity to Edit Mischarged tRNA^{Phe}—To test the capacity of yeast mitochondrial PheRS to edit misaminoacylated products, Tyr-tRNA^{Phe} was incubated with the enzyme under conditions previously used to monitor the same activity in bacterial and cytosolic enzymes. No significant hydrolysis of Tyr-tRNA^{Phe} was observed in the presence of excess enzyme, the rate of deacylation being comparable with that seen in the absence of PheRS (Fig. 5*A*). These data showed that yeast mitochondrial PheRS, in contrast to its cytosolic counterpart, was unable to *trans* edit exogenous Tyr-tRNA^{Phe}. To probe the possibilities that mitochondrial PheRS either edits solely at the pretransfer step or that it edits Tyr-tRNA^{Phe} only in *cis* (*i.e.* when still bound to PheRS), we attempted to tyrosylate tRNA^{Phe} with the wild-type enzyme. Wild-type mitochondrial PheRS was able to stably synthesize Tyr-tRNA^{Phe}, suggesting the absence of effective editing mechanisms in this enzyme (Fig. 5*B*). To investigate whether mitochondrial PheRS contains a low level of *cis* editing activity not readily detectable under the standard assay conditions, the amino acid binding pocket was enlarged to better accommodate tyrosine as described previously (45) for the *E. coli* enzyme. The resulting variant, mitochondrial PheRS A333G, showed considerably improved Tyr-tRNA^{Phe} synthesis compared with the wild type providing further evidence that the organellar protein lacks editing activity (Fig. 5*B*). We also attempted to monitor pretransfer editing as described previously (25) for the *E. coli* enzyme but were unable to detect any significant activity for either the cytosolic or mitochondrial enzymes (data not shown).

Trans Editing of Tyr-tRNA^{Phe} Is Confined to Cytosolic Protein Synthesis in Yeast—Although our *in vitro* studies showed that mitochondrial PheRS could not edit Tyr-tRNA^{Phe}, it is possible that other factors might act in *trans* after dissociation of the non-cognate aa-tRNA from the enzyme. Such factors could include specific *trans* editing enzymes (24, 46) or cytosolic PheRS imported into yeast mitochondria as described for other aaRSs (37). Yeast mitochondrial and cytosolic fractions were purified as described previously (37), and the localization of PheRS was examined in each compartment by immunoblotting (Fig. 6*A*). Cytosolic PheRS was not detectable in mitochondrial extracts, suggesting that *trans* editing by imported β-subunits would not compensate for the absence of editing activity in the mitochondrial enzyme. The amount of each subcellular fraction required to aminoacylate comparable amounts of tRNA^{Phe} with Phe was quantified (Fig. 6*B*), and based upon these measurements comparable phenylalanylation activities were then incu-

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TABLE ONE

Steady-state kinetic constants for ATP-[^{32}P]PPi exchange for cytosolic and mitochondrial PheRS from yeast

PheRS	Phe			Tyr			Selectivity $(k_{cat}/K_M)_{Phe}/(k_{cat}/K_M)_{Tyr}$
	K_M	k_{cat}	k_{cat}/K_M	K_M	k_{cat}	k_{cat}/K_M	
	μM	s^{-1}	$s^{-1}\ \mu M^{-1}$	μM	s^{-1}	$s^{-1}\ \mu M^{-1}$	
Cytosol	30 ± 2	240 ± 40	8 ± 2	860 ± 300	160 ± 40	0.2 ± 0.1	40 ± 30
Mitochondria	4.2 ± 0.1	150 ± 5	40 ± 2	(1700)[a]	(150)[a]	0.09 ± 0.01[b]	400 ± 80

[a] Lower limits based on the assumption that the mitochondrial enzyme, like the cytoplasmic enzyme, has similar k_{cat} values for Tyr and Phe.
[b] Due to the high K_M compared to practical Tyr concentrations ($[S] \ll K_M$), k_{cat}/K_M was directly estimated from the equation $v = k_{cat}/K_M\ ([E][S])$.

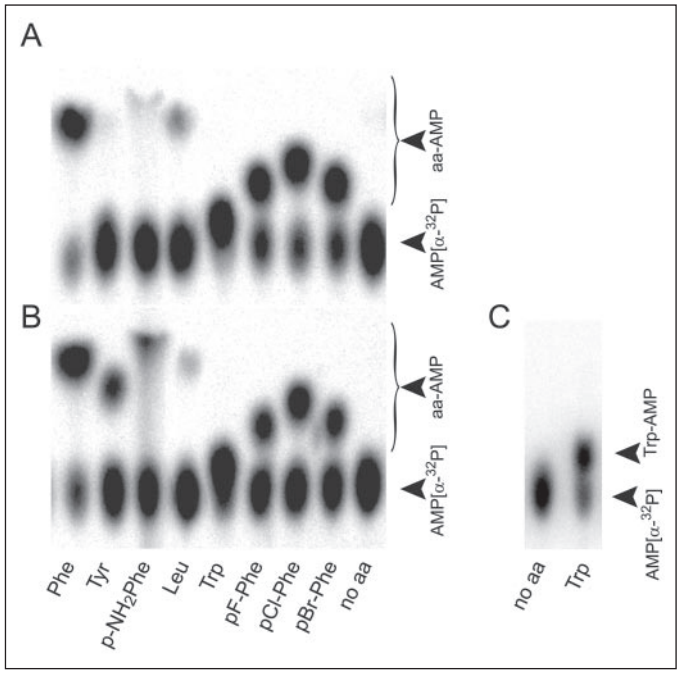

FIGURE 4. **Substrate specificity of editing by yeast cytosolic PheRS.** TLC analysis of the aminoacylation of tRNA^{Phe} with various amino acids and Phe analogues by the wild-type cytosolic PheRS (*A*) and the editing-defective variant βD243A PheRS (*B*). TLC analysis of the aminoacylation of tRNA^{Trp} with Trp by tryptophanyl-tRNA synthetase (*C*). *No aa*, no amino acid.

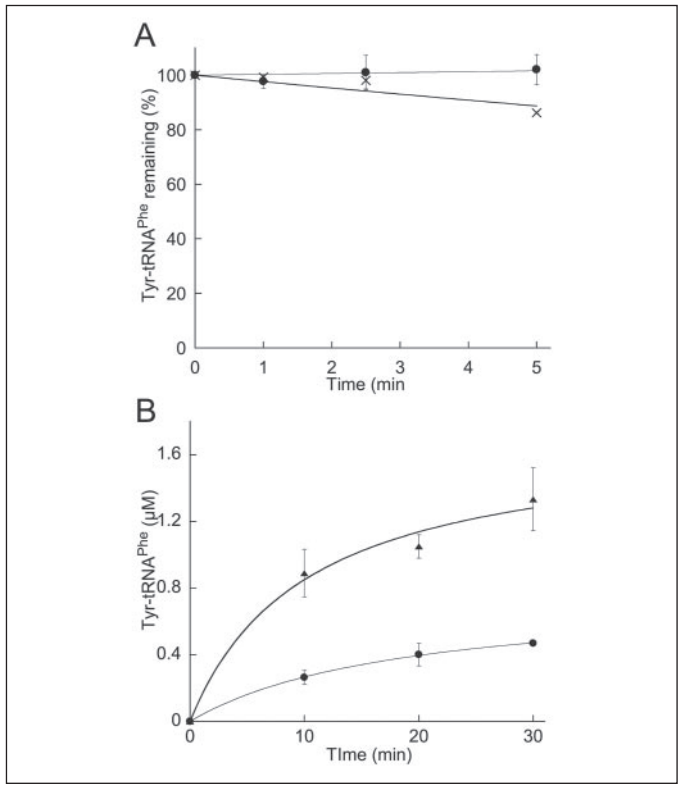

FIGURE 5. **Non-cognate aminoacylation by yeast mitochondrial PheRS.** *A*, deacylation of Tyr-tRNA^{Phe}. Tyr-tRNA^{Phe} was prepared using *in vitro* transcribed yeast mitochondrial tRNA^{Phe} and incubated in the presence of 1 μM (●) yeast mitochondrial PheRS or without enzyme (×). *B*, aminoacylation of the mitochondrial tRNA^{Phe} transcript (3.5 μM) with Tyr (30 μM) by the mitochondrial PheRS (2 μM). ●, wild-type mitochondrial PheRS; ▲, A333G mitochondrial PheRS. The comparatively low final charging level of tRNA (10–30%) likely reflects reactions performed at substantially sub-saturating concentrations of Tyr because of practical considerations.

bated with different Tyr-tRNA^{Phe} species and the rate of deacylation monitored (Fig. 6*C*). The cytosolic fraction was able to rapidly deacylate Tyr-tRNA^{Phe}, whereas the mitochondrial extract showed no significant difference in the rates of deacylation of Phe-tRNA^{Phe} and Tyr-tRNA^{Phe}. These findings were in agreement with the *in vitro* data described above and supported the notion that Tyr-tRNA^{Phe} is not subject to proofreading and editing in yeast mitochondria.

Phylogenetic Analysis of PheRS—Phylogenetic trees were calculated using two neighbor-joining methods and a character-based maximum likelihood method and rooted with an alignment of *o*-phosphoseryl-tRNA synthetase sequences. All trees exhibited the same overall topology (Fig. 7), with the mitochondrial/eubacterial sequences forming one major cluster and archaeal/eukaryotic sequences the other. In agreement with previous reports (47, 48), the only exceptions were spirochete PheRS sequences, which clustered with the archaea, and mycoplasmataceae PheRS sequences, which clustered with the mitochondria.

DISCUSSION

Yeast Mitochondrial PheRS Is an Error-prone Enzyme—Previous studies (26) have shown that although the two PheRSs of yeast differ significantly in their tertiary and quaternary structures, they share similar kinetic parameters for cognate aminoacylation and are thus functionally analogous. The most significant difference between the cytosolic and mitochondrial versions of human PheRS is a lower apparent affinity for tRNA in the organellar enzyme (27). High-resolution crystal structures suggested that the substantial differences between the canonical $(\alpha\beta)_2$ PheRS and the mitochondrial α-type might not significantly impact tRNA aminoacylation, as the additional domains in the former were presumed to have alternative non-canonical functions. For example, the helix-turn-helix motif in domain B5 was shown to be a *bona fide* DNA binding motif in *T. thermophilus* PheRS, capable of binding looped double-stranded DNA (49). Deletion and mutagenesis of B5 and other domains did not abolish the canonical activity of PheRS, leading to the conclusion that domains 1–5 of the β-subunit are dispensable for efficient aminoacylation (50). One notable exception is the B3/B4 editing domain, which is required *in vitro* and *in vivo* for the hydrolysis of non-cognate Tyr-tRNA^{Phe} synthesized by PheRS. No region homologous to B3/B4 is detectable at the amino acid sequence level in mitochondrial PheRS, and the studies described here show that this correlates with a lack of editing activity in the enzyme. Comparison

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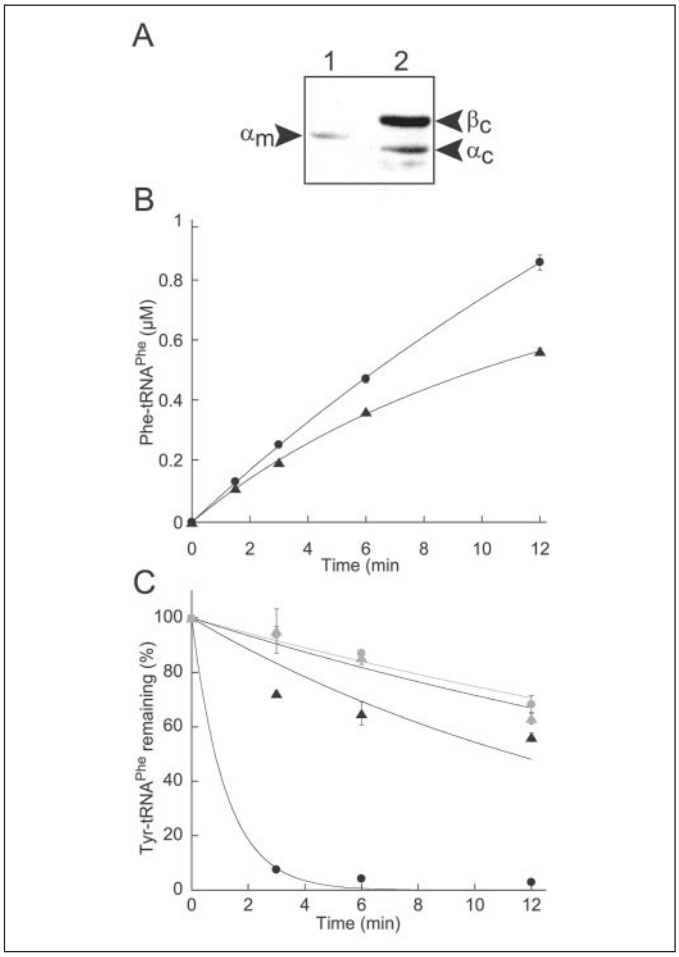

FIGURE 6. **Aminoacylation and post-transfer editing by yeast mitochondrial and cytosolic fractions.** *A*, immunodetection of PheRS in cytosolic and mitochondrial fractions. 20 μg of protein from mitochondrial and cytosolic fractions was subjected to SDS/PAGE, and the PheRSs were subsequently detected by immunoblotting using rabbit anti-yeast cytosolic PheRS antibodies. This allowed detection of mitochondrial PheRS (α_m) and both subunits of the cytosolic PheRS (α_c and β_c) as indicated by the corresponding *arrows*. *Lanes*: *1*, mitochondrial fraction; *2*, cytosolic fraction. *B*, phenylalanylation activity. Aminoacylation of the cytosolic tRNA^{Phe} transcript (5 μM) was performed with Phe (30 μM) by the mitochondrial (160 μg of protein/ml) (▲) or cytosolic (180 μg of protein/ml) (●) fractions. *C*, post-transfer editing of Tyr-tRNA^{Phe}. Tyr-tRNA^{Phe} (*black*) or Phe-tRNA^{Phe} (*gray*) was prepared using *in vitro* transcribed yeast cytosolic or mitochondrial tRNA^{Phe} and incubated, respectively, with the same amounts of cytosolic (*circles*) or mitochondrial (*triangles*) crude extracts as used in *B*.

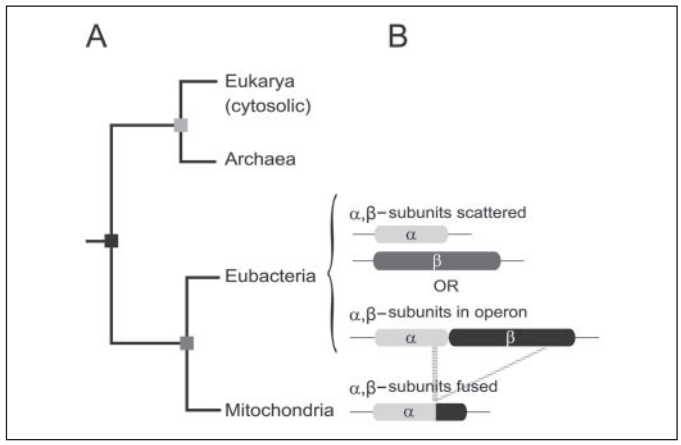

FIGURE 7. **Phylogenetic analysis of PheRS α-subunits (*A*) and comparison of the organization of eubacterial and mitochondrial PheRS genes (*B*).** *Black squares*, *dark gray squares*, and *light gray squares* indicate nodes found in >95%, >85%, and >65%, respectively, of the trees generated by neighbor-joining or maximum likelihood analysis. A single recombination event (*dashed line*) involving the eubacterial α,β-subunit operon might be at the origin of the fused α,β mitochondrial PheRS.

of Phe and Tyr activation by the cytosolic and mitochondrial enzymes showed only modest differences in substrate specificities, indicating that both enzymes have significant capacities for non-cognate aminoacylation. Previous studies (51) have shown that Tyr and Phe are likely present at comparable levels in mitochondria and the cytosol, indicating that the likelihood of non-cognate amino acid activation by PheRS will not be affected by subcellular localization. For the cytosolic enzyme, the existence of an editing mechanism ensures that any errors in amino acid activation do not affect the overall accuracy of the aminoacylation reaction. No such editing mechanism exists, either in *cis* or *trans*, during mitochondrial phenylalanylation, and the organellar PheRS can be considered an error-prone enzyme. Other aaRSs, such as certain ProRSs (46, 52) and ThrRSs (21), have also been shown to have natural uncorrected mischarging activities that result in non-cognate aminoacyl-tRNA synthesis. However, in both cases the enzymes are not considered error-prone, as they associate with other proteins that ensure non-cognate aminoacyl-tRNAs are rapidly edited (53). Mitochondrial PheRS is markedly different in that no detectable editing activity is associated with it, suggesting amino acid specificity was lost during evolution from more accurate ancestral proteins.

Loss of the Editing Subdomain during Evolution of Mitochondrial PheRS—Previous analyses (47, 48) based upon alignments of PheRS α-subunit amino acid sequences indicated a canonical phylogeny for this protein family, with the only significant horizontal gene transfer being observed in the spirochetes. The updated analysis described here provides a similar phylogenetic tree composed of monophyletic archaeal/eukaryotic and bacterial clusters, with the mitochondrial PheRSs forming a single, distinct subgroup within the latter (Fig. 7). These data suggest a single bacterial origin for extant mitochondrial PheRSs but provide few clues on the subsequent evolution of the organellar protein. The widespread retention of a discrete full-length β-subunit indicates that editing activity was lost after separation of the mitochondrial proteins. In addition to the absent B3/B4 editing module, mitochondrial PheRSs lack the B5 DNA binding domain, B6/B7 oligomerization domain, and B2 EMAP domain, suggesting that loss of editing may have been part of a broader process whereby numerous functions dispensable for protein synthesis were lost during evolution of the organellar protein. This loss of function is, to date, limited to mitochondria, as chloroplast PheRSs are of the $(\alpha\beta)_2$ type and contain an intact B3/B4 domain (54).

Editing of Misacylated tRNAs in Mitochondria—The loss of editing function from mitochondrial PheRS is particularly striking because it was accompanied by a significant change in the oligomeric state of the protein. Less dramatic changes were also shown to lead to a loss of editing function from human mitochondrial LeuRS, which is able to stably synthesize Ile-tRNA^{Leu} *in vitro* (55). The editing function of LeuRS is contained in the highly conserved CP1 domain (56, 57), but key residues in this region necessary for hydrolysis of non-cognate aminoacyl-tRNA have diverged during evolution leading to a loss of function from the mitochondrial protein. Although it is unclear whether other factors act in *trans* to compensate for the lack of editing by mitochondrial LeuRS, the evolutionary trend toward a more error-prone aaRS mirrors that described above for PheRS. This, in turn, raises the question as to whether other mitochondrial aaRSs might also lack the capacity to edit misaminoacylated tRNAs. For example, mitochondrial ThrRS lacks either of the modules known to edit Ser-tRNA^{Thr} (19–21) suggesting that this misacylated species might also escape hydrolysis. However, Ser-tRNA^{Thr} can be directly hydrolyzed by free-standing editing

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domains acting in *trans* emphasizing the need for direct experimental confirmation that particular editing pathways are absent from mitochondria.

The absence of certain aaRS editing reactions from mitochondria suggests that the need for fidelity during aminoacyl-tRNA synthesis may be lower in this compartment than in the cytosol, perhaps accelerating the loss of these energetically costly secondary activities. Loss of the editing domain from mitochondrial PheRS is in stark contrast to the general pattern of synthetase evolution, where modules with new functionality have been systematically integrated into extant proteins (58). This fundamental difference between mitochondrial and cytosolic aaRS evolution and function indicates that either organellar protein synthesis quality control is focused on another step or that translation in this compartment is inherently less accurate. From a physiological standpoint, reduced accuracy during translation might not be as detrimental in the mitochondrial system, where only a small number of proteins are encoded. In the presence of an efficient protein degradation machinery, it is not unlikely that the bulk of the mitochondrial quality control occurs post-translationally at the level of protein stability as has been recently shown (see for example Ref. 59 and references therein). In this respect, only folded proteins that can join an active respiratory complex are protected from degradation. Mistakes in any protein subunit resulting from reduced translational accuracy can be easily accommodated by very rapid protein degradation. This model could be easily re-enforced by measuring translational accuracy with mitochondrial ribosomes and comparing the error rate to that of degradation. However, currently *in vitro* translation systems with mitochondrial ribosomes have not been established, and this will thus remain an open question.

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Loss of Editing Activity during the Evolution of Mitochondrial Phenylalanyl-tRNA Synthetase

Hervé Roy, Jiqiang Ling, Juan Alfonzo and Michael Ibba

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