Supplemental data for

Kinetic partitioning between synthetic and editing pathways in class I aminoacyl-tRNA synthetases occurs at both pre-transfer and post-transfer hydrolytic steps

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

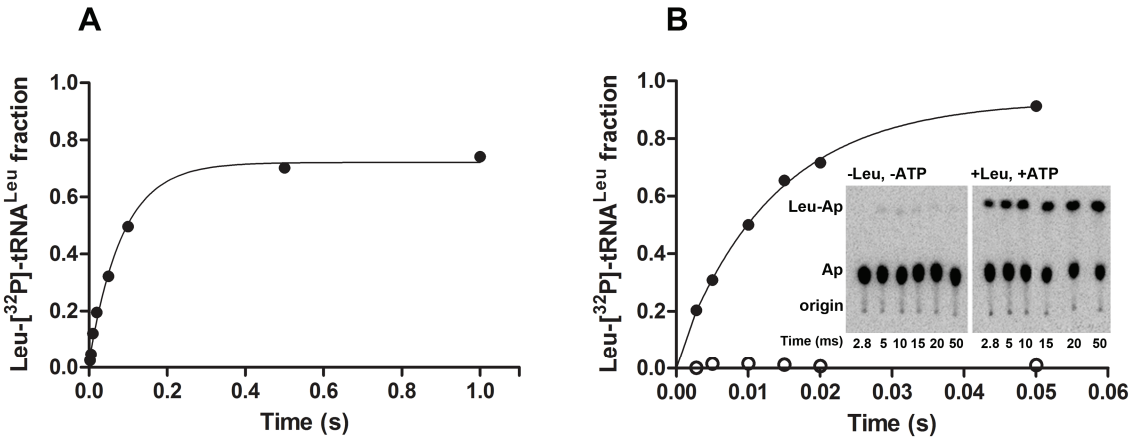
Additional purification step for enzymes used in single-turnover kinetics and preparative misacylation reactions

Control reactions to assess whether purified WT and mutant *E.coli* LeuRS contain bound Leu-AMP were performed by mixing 1 μM [^{32}P]-tRNA^{Leu} with 10 μM WT LeuRS in 100 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 1 mM DTT and 10 μg/ml BSA in the absence of both ATP and leucine. Reaction aliquots were quenched in a mixture of NaOAc (pH 4.5, 0.8 M final concentration) and SDS (final w/v of 0.1%) followed by tRNA hydrolysis using P1 nuclease (final concentration 0.1 mg/ml). TLC analysis was performed as described previously (1, 2; see Supplemental Fig. 1A).

Removal of Leu-AMP from purified LeuRS was accomplished by incubating enzyme preparations with tRNA^{Leu} (molar ratio 2:1) in 100 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 150 mM KCl, 1 mM DTT and 10 μg/ml BSA for 15 min at 37 °C, thus allowing formation of Leu-tRNA^{Leu} with release of AMP. To separate the aminoacylated tRNA from LeuRS, the reaction mixture was first incubated in a solution containing 2 M urea and 1 M NaCl for 1 h on ice. Subsequently, LeuRS was repurified from the mixture in the presence of 2 M urea and 1 M NaCl by binding and elution from Ni^{2+}-NTA resin, and then dialyzed extensively against 20 mM Tris-Cl (pH 7.5), 50 mM NaCl and 10% glycerol prior to storage at -80 °C. Removal of tRNA was assessed by measuring the A_{260}/A_{280} ratio of the purified enzyme. Control reactions in single-turnover conditions performed with LeuRS purified in this manner, revealed less than 1% Leu-[^{32}P]-tRNA^{Leu} formation in the absence of amino acid and ATP (see Supplemental Fig. 1B).

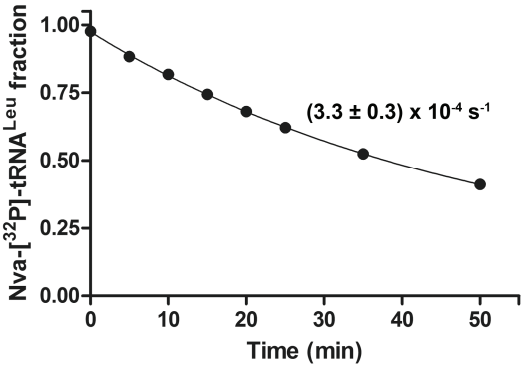
It must be noted that comparisons of steady-state activities show that *Ec*LeuRS variants lose approximately 25% of their activities during this procedure. However, this is not a concern because the purified variant is necessary only in reactions in which the enzyme concentration is comparable or higher than tRNA concentration. All single-turnover reactions were performed under conditions of enzyme saturation in which the lower activity of purified variants had no effect on the reaction rate (k_{trans} for leucine was the same with purified and unpurified variants).

Supplemental Figure 1.



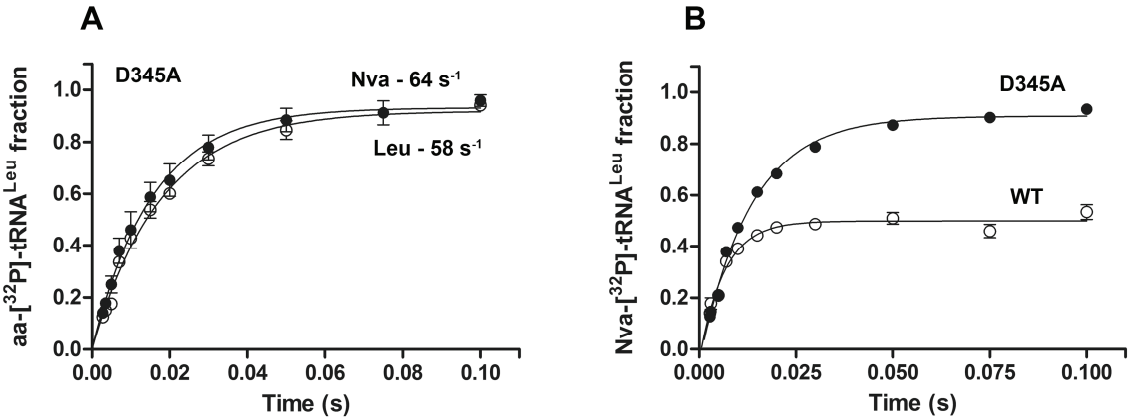
Copurification of *Ec*LeuRS with Leu-AMP. *A*, Leu-[^{32}P]-tRNA^{Leu} formation by 10 μM WT LeuRS in the presence of 1 μM [^{32}P]-tRNA^{Leu} and absence of leucine and ATP in the reaction mixture. The high plateau of Leu-tRNA^{Leu} formation under these conditions demonstrates that LeuRS copurifies with Leu-AMP. *B*, control transfer reactions performed with 1 μM [^{32}P]-tRNA^{Leu} and 10 μM WT LeuRS from which endogenous Leu-AMP was removed, in the presence (●) or absence (○) of saturating concentrations of leucine and ATP in the reaction mixture. The inset shows thin layer chromatograms that correspond to reaction progress curves illustrated on the graph. Leu-Ap indicates the position where leucylated A76 nucleotide migrates, while Ap indicates the position where the non-aminoacylated 3'-terminal monophosphorylated A76 nucleotide migrates. LeuRS from which Leu-AMP has been removed forms almost no detectable Leu-tRNA^{Leu} in reactions without ATP and leucine, demonstrating that our purification procedure yields LeuRS essentially free of Leu-AMP. Additionally, the same k_{trans} for leucine transfer to tRNA^{Leu} was obtained with both purified and unpurified LeuRS variants in single-turnover aminoacyl transfer reactions.

Supplemental Figure 2.



Nonenzymatic deacylation of Nva-[^{32}P]-tRNA^{Leu}. The fraction of Nva-[^{32}P]-tRNA^{Leu} (●) remaining in solution was plotted as a function of time. A fit to a first-order exponential equation provided a rate constant of 3.3 x 10^{-4} s^{-1}. Rates of nonenzymatic aa-tRNA^{Leu} hydrolysis used for correction of enzyme-catalyzed deacylation rates (see Table 2) were obtained under the same conditions and the same time frame as the enzyme-catalyzed reaction (from 3 to 12 minutes). These reaction times were chosen to satisfy steady-state conditions for enzymatic hydrolysis of aa-tRNA^{Leu}. On this shorter time scale, the rate of nonenzymatic hydrolysis was apparently constant (1.9 nM s^{-1}), while on a longer time scale exponential decay with a rate constant of 3.3 x 10^{-4} s^{-1} was observed.

Supplemental Figure 3.



A) Single-turnover transfer of norvaline (●) or leucine (○) by D345A *Ec*LeuRS.
B) Accumulation of Nva-tRNA^{Leu} under rapid chemical quench conditions by WT (○) and D345A (●) LeuRS enzymes. Reaction conditions are the same as used for determination of the transfer step (see Experimental Procedures: Single-turnover transfer step). In both cases, tRNA^{Leu} was present at 1 μM concentration, and LeuRS:aa-AMP (formed *in situ*) was present at 10 μM concentration. WT LeuRS displays plateau formation of Nva-tRNA^{Leu}, because the reaction conditions used (saturating concentrations of ATP and Nva) enable multiple turnovers by rapid reformation of Nva-AMP, and regeneration of tRNA^{Leu} through deacylation in the CP1 editing site. In contrast, when single-turnover conditions are applied (ensured by the limiting amounts of ATP), Nva-tRNA^{Leu} formation and deacylation is observed as shown for T252R LeuRS (Fig. 6).

Supplemental Table 1. Nonenzymatic aa-AMP hydrolysis

aa-AMP	k_{obs}
	s^{-1}
Nva-AMP[a]	$(14 \pm 3) \times 10^{-4}$
Leu-AMP[b]	$(17 \pm 9) \times 10^{-4}$

The values represent the best fit value ± s.e.m. of [a]4 or [b]2 independent experiments.

Supplemental Table 2. Steady-state parameters for Nva-AMP formation[a]

***Ec*LeuRS**	**-tRNA**			**+ tRNA**		
	K_m **(Nva)**	k_{cat}	k_{cat}/K_m	K_m **(Nva)**	k_{cat}	k_{cat}/K_m
	mM	s^{-1}	$mM^{-1}\ s^{-1}$	mM	s^{-1}	$mM^{-1}\ s^{-1}$
WT	3.8 ± 0.9	0.052 ± 0.003	0.014	n.d.[b]	n.d.	n.d.
D345A	4 ± 1	0.043 ± 0.004	0.011	7 ± 2	0.050 ± 0.003	0.007
T252R/D345A	4.3 ± 0.7	0.062 ± 0.003	0.014	4.7 ± 0.7	0.061 ± 0.004	0.013

[a] Measured by aminoacyl-adenylate synthesis assay.
The values represent the best fit value ± s.e.m. of three independent experiments.
The enzymes were assayed at 0.5 μM concentration and norvaline concentration was varied over the range 0.1-10 times the K_m.
[b] n.d. – not determined due to activity too low for reliable detection.

SUPPLEMENTAL REFERENCES

1. Gruic-Sovulj, I., Uter, N., Bullock, T., and Perona, J. J. (2005) tRNA-dependent aminoacyl-adenylate hydrolysis by a nonediting class I aminoacyl-tRNA synthetase. *J. Biol. Chem.* **280**, 23978-23986
2. Dulic, M., Cvetesic, N., Perona, J. J., and Gruic-Sovulj, I. (2010) Partitioning of tRNA-dependent editing between pre- and post-transfer pathways in class I aminoacyl-tRNA synthetases. *J. Biol. Chem.* **285**, 23799-23809