Supplemental Information

Amino acid-dependent Shift in tRNA Synthetase Editing Mechanisms

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Materials: Radiolabeled amino acids were purchased from Amersham Pharmacia Biotech (Piscataway, NJ) or Perkin Elmer (Waltham, MA). [α^{32}P]-ATP was acquired from Perkin Elmer (Waltham, MA). Crude *Saccharomyces cerevisiae* tRNA was acquired from Sigma Chemical Co. (St Louis, MO).

Protein purification: The plasmid p32YL-2-3[1] encoding the gene for the wild type yeast cytoplasmic leucyl-tRNA synthetase (ycLeuRS) was used to express the protein in *E. coli* strain Rosetta (DE3) (Novagen). A single transformant was used to inoculate 5 mL LB that contained 100 μg/mL ampicillin and 34 μg/mL chloramphenicol, which was then incubated at 37 °C overnight. The overnight culture was used to inoculate 500 mL LB (100 μg/mL ampicillin and 34 μg/mL chloramphenicol) and cells were grown at 30 °C until the OD_{600} reached 0.4-0.6. At this stage, cells were induced with 1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG, Sigma-Aldrich) and the protein was expressed at 30 °C for 45 min. Cells were harvested using an Avanti J-E preparative centrifuge (Beckman Coulter, Fullerton, CA), at 6000 rpm for 15 min at 4 °C.

Cell pellets were resuspended in 20 ml of HA-I buffer [20 mM Na_2HPO_4, 10 mM tris(hydroxymethyl) aminomethane (Tris), pH 8.0, 100 mM NaCl and 5% glycerol]. This was followed by sonication on ice for 2 min at 50% amplitude and a 3 sec pulse using a

Vibra Cell sonicator (Sonics, Newtown, CT). The 2 min cycle of sonication was repeated three times. The cell lysate was centrifuged at 12,000 rpm for 30 min at 4 °C. The protein-containing supernatant was loaded onto a prepacked Ni Sepharose high performance 5 mL column (HisTrap HP, GE Healthcare Biosciences, Uppsala, Sweden) that had been pre-equilibrated with 30 mL of HA-I buffer. Following binding of the N-terminal six-histidine tagged LeuRS, the column was washed with 100 mL of HA-II buffer [20 mM Na_2HPO_4, 10 mM Tris, pH 7.0, 500 mM NaCl and 5% glycerol] containing 10 mM imidazole at 4 °C. The bound protein was then eluted in fractions from the resin using an immidazole gradient of 10 to 100 mM in HA-I. The LeuRS containing fractions were pooled and concentrated using an Amicon Ultra centrifugal filter device (Millipore Corporation, Billerica, MA). The final protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 155,310 $M^{-1}cm^{-1}$, estimated by the ExPASy Protparam tool (http://ca.expasy.org/tools/protparam.html).

Cloning of the gene encoding yeast cytoplasmic $tRNA^{Leu}_{CAA}$ ($yctRNA^{Leu}$): The gene fragment encoding the $yctRNA^{Leu}_{CAA}$ was amplified in a 50 µL polymerase chain reaction (PCR) that contained 100 ng of pAMWt-25 template plasmid DNA, 125 ng each of forward [pTRC-Fw(*EcoRI*)-$yctRNA^{Leu}$] and reverse [pTRC-Rv(*BamHI*)-$yctRNA^{Leu}$] primer containing the *Eco*RI and *Bam*HI restriction sites respectively, 0.05 mM dNTP mix and 0.05 U *Pfu* DNA polymerase in commercial buffer. The PCR products were digested with *Eco*RI and *Bam*HI at 37 °C for 6 hr. The restriction-digested PCR products were separated on a 2% agarose gel and gel-purified using the QIAquick gel extraction kit-250 (Qiagen Inc). The vector pTrc-99[2] (a gift from G. Eriani) was also cleaved with

*EcoR*I and *Bam*HI, followed by gel-extraction. Gel purified restriction digests of the PCR amplified gene fragment and the vector were ligated using T4 DNA ligase at 37 °C for 15 min to yield the plasmid pJS-pTrc-(*EcoRI*)-yctRNA$^{Leu}_{CAA}$-(*BamHI*) The ligation reaction was used to transform *E. coli* strain DH5α. The gene sequence was confirmed by automated DNA sequencing (UIUC Core Sequencing Facility, Urbana).

Expression and purification of yctRNA$^{Leu}_{CAA}$: The yctRNA$^{Leu}_{CAA}$ was overexpressed in *E. coli* strain DH5α harboring the plasmid pJS-pTrc-(*EcoRI*)-yctRNA$^{Leu}_{CAA}$-(*BamHI*). A single transformant was used to inoculate 6 mL LB (100 μg/mL ampicillin) which was incubated at 37 °C overnight. The overnight culture was used to inoculate 2 L LB (100 μg/mL ampicillin) and cells were grown at 37 °C until the OD_{600} reached 0.6-0.8. At this stage, cells were induced with IPTG, to a final concentration of 1 mM and the yctRNA$^{Leu}_{CAA}$ was expressed overnight at 37 °C. Cells were harvested with an Avanti J-E preparative centrifuge (Beckman Coulter, Fullerton, CA), at 6000 rpm for 15 min at 4 °C.

Cell pellets were resuspended in 50 mL of buffer [20 mM Tris, pH 7.5; 20 mM magnesium acetate]. This was followed by two extractions with 50 mL each of phenol/Tris saturated solution (Acros Organics, New Jersey, USA) and vigorous shaking for 20 min at room temperature. The aqueous phase was collected after clarification via centrifugation at 3000 rpm for 10 min at 10 °C and then ethanol precipitated at - 80 °C overnight. The RNA was collected by centrifugation, followed by resuspension of the pellet in 500 mM Tris, pH 8 at 37 °C to deacylate the tRNA. The tRNA was then purified on a 10% denaturing polyacrylamide gel by electrophoresis. The concentration

of tRNA was measured spectrophotometrically on the basis of its extinction co-efficient of 815,300 $M^{-1}cm^{-1}$ estimated by the online Ambion oligonucleotide calculator (http://www.ambion.com/techlib/misc/oligo_calculator.html)

Misaminoacylation assays: Each misaminoacylation reaction contained 60 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 30 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol (DTT), 4 μM pure yctRNA^{Leu}_{CAA}, 25 μM [^{3}H]-methionine (422 μCi/mL) and 1 μM enzyme. Reactions were initiated with 4 mM ATP. At various time points, 10 μL reaction aliquots were quenched on Whatman filter pads that had been pre-wet in 5% trichloroacetic acid (TCA) and then dried. The pads were then subjected to the following 10 min washes: three times with 5% TCA and once with cold 70% ethanol. The pads were dried under a heat lamp and then quantitated for radioactivity in a liquid scintillation counter (Beckman LS 6500, Beckman Coulter, Fullerton, CA). GraphPad Prism software was used to plot data.

Isolation of mischarged $tRNA^{Leu}$. Purified yctRNA^{Leu}_{CAA} (8 μM) was misaminoacylated with isoleucine or methionine by incubating the following reaction mixture at 30 °C for 3 h in 60 mM HEPES, pH 7.5, 30 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 23.8 μM [^{3}H]-isoleucine (422 μCi/mL) or 25 μM [^{3}H]-methionine (422 μCi/mL), 1 μM editing defective (D419A) ycLeuRS and 4 mM ATP. The reaction was quenched under acidic conditions with 0.18% acetic acid[3] to stabilize the aminoacyl ester linkage between isoleucine and the terminal adenosine (A76) on $tRNA^{Leu}$. Protein was removed from the reaction mixture by phenol extraction using a 125:24:1 phenol:choloroform:isoamyl alcohol mixture, pH 4.3 (Fisher Scientific, Fair Lawn, NJ). Mischarged tRNA was ethanol precipitated at -80 °C overnight in the presence of 0.34

mg/mL glycogen. The recovered tRNA pellet was washed twice with 70% ethanol, dried, and resuspended in 50 mM KH_2PO_4, pH 5.0. For each reaction, a total of six 2 μL duplicate aliquots were spotted on TCA-soaked and dried pads. Half of the pads were washed extensively (as described above) to quantitate the [^{3}H]-Ile-tRNA^{Leu} and [^{3}H]-Met-tRNA^{Leu} mischarged products[4].

Deacylation assays. Deacylation reaction mixtures were carried out at pH 7.5 in 60 mM HEPES, 10 mM $MgCl_2$, 10 mM KCl and approximately ~2 μM [^{3}H]-Ile-tRNA^{Leu} or [^{3}H]-Met-tRNA^{Leu}. The reactions were initiated with 1 μM enzyme. At desired time points, reaction aliquots of 10 μL were quenched on Whatman filter pads that had been pre-wet in 5% TCA and dried. Pads were washed and then quantified for radioactivity as described above.

AMP formation assay. The formation of AMP was carried out at 30 °C in a reaction mixture containing 50 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 20 mM KCl, 5 mM DTT, 0.024 U/μL inorganic pyrophosphatase, 250 μM cold ATP, 0.2 μM [α-^{32}P]-ATP (600 μCi/mL), 2.5 mM leucine or 100 mM isoleucine or 100 mM methionine. Reactions were also measured for their dependence on tRNA by incorporating 29 mg/mL crude yeast tRNA, which was measured to contain 14.5 μM tRNA^{Leu}. Reactions were initiated with 1 μM enzyme. At different time points, reaction aliquots (2.0 μL) were quenched in 8.0 μL of 200 mM sodium acetate, pH 5.0. From each quenched aliquot, 3 μL was spotted on cellulose polyethyleneimine (PEI) thin layer chromatography (TLC) plates (Scientific Adsorbents Inc., Atlanta, GA) that were pre-run in water and dried. Separation of [α-^{32}P]-ATP, [^{32}P]-AMP and [^{32}P]-AA-AMP were achieved by developing the TLC plates in 0.1 M ammonium acetate, 5% acetic acid[5]. Resolved radiolabeled

bands on the TLC plate were phosphorimaged using FUJIFILM BAS Cassette 2040 (FUJIFILM Medical Systems, Stanford, CT) and images were scanned by a STORM 840 Molecular Dynamics scanner (Amersham Pharmacia Biotech, Piscataway, NJ). At each time point, the remaining ATP was measured as a fraction, of the total volume intensity of all the resolved spots at that time point. A known concentration of [α-^{32}P]-ATP was spotted on each TLC plate. The concentration of AMP in each separated band was calculated by comparing the volume density for each [^{32}P]-AMP spot to the volume density of the known [α-^{32}P]-ATP spot. Images were quantified using the ImageQuant software and the resulting data plotted and analyzed using the GraphPad Prism software. The observed rate constants were calculated from a linear fit of the respective data sets.

Nonenzymatic hydrolysis of AA-AMP. The rate of nonenzymatic hydrolysis of AA-AMP was calculated using an adapted chase assay[6] where a molar excess of cold ATP (25 mM) is used to eject the AA-AMP from the enzyme's active site. Enzyme synthesized AA-AMP was allowed to form and accumulate for 10 min at 30 °C in a reaction mixture containing 50 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 20 mM KCl, 5 mM DTT, 250 μM cold ATP, 0.6 μM [α-^{32}P]-ATP (1800 μCi/mL), 100 mM methionine and 5 μM enzyme. Following addition of molar excess of ATP (100 fold), hydrolysis of the AA-AMP in solution was quenched at various time points by mixing 2.0 μL reaction aliquot with 8.0 μL of 200 mM sodium acetate, pH 5.0. Separation of reaction products on TLC plates were performed and quantified as described above. The rate constants for AA-AMP hydrolysis in solution was calculated by fitting the respective data sets to the equation $y = Ae^{-kx}$, using GraphPad Prism software.

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