Supporting Information: JA0177096

Plasmid Construction

*E. coli pheS** was amplified by the polymerase chain reaction (PCR) from vector pQE-FS. Amplified *pheS** was subjected to PCR mutagenesis to create the coding sequence for the desired Thr251Gly mutant, which we designate *pheS***. To allow constitutive expression of the synthetase, a linker encoding a *tac* promoter with an abolished *lac* repressor binding site was prepared with terminal *Nhe*I restriction sites and internal *Nco*I and *Hind*III sites. The linker sequence is

5'CTAGCAG<u>TTGACAA</u>TTAATCATCGGCTCG<u>TATAATG</u>GATCGAATTGT GAGCGGAATCGATTTTCACACAGGAAACAGACC**ATG**GATCTTCGTCGCCATC CTCGGGTCGACGTCTGTTTGCAAGCTTG-3'

(the –35 and –10 sequences are underlined and start codon is in bold). This linker was cloned into the *Nhe*I site of vector pET5a (Novagen) to form pET5a-tac. PCR amplified fragments containing *pheS** and *pheS*** were cloned into pET5a-tac at the *Nco*I and *Hind*III sites. *pheS** and *pheS*** outfitted with the *tac* promoter were cut out as *Nhe*I fragments and inserted into expression plasmid pQE15 (Qiagen) to yield pQE-FS* and pQE-FS** respectively. Expression plasmids pQE15, pQE-FS* and pQE-FS** encode murine dihydrofolate reductase (mDHFR) under control of a bacteriophage T5 promoter.

Determination of Translational Activity

Buffer and media were prepared according to standard protocols. A phenylalanine auxotrophic derivative of *E. coli* strain BL21(DE3), designated AF (*HsdS gal* (*λc*I*ts*857 *ind* 1 *S*am7 *nin*5 *lac*UV5-T7 gene *1*) *pheA*) and constructed in our laboratory, was used as the expression host. The AF strain was transformed with repressor plasmid pLysS-IQ and with pQE15, pQE-FS* or pQE-FS** to afford expression strains AF-IQ[pQE15], AF-IQ[pQE-FS*] or AF-IQ[pQE-FS**] respectively.

Small scale (10 ml) cultures were used to investigate the *in vivo* translational activity of **2**. M9 minimal medium (50 ml) supplemented with 0.2 % glucose, 1mg/L thiamine, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 19 amino acids (at 20 mg/L), antibiotics (ampicillin 200 mg/L, chloramphenicol 35 mg/L) and phenylalanine (at 20 mg/L) was inoculated with 1 ml of an overnight culture of the expression strain. When the optical density at 600 nm reached 0.8-1.0, a medium shift was performed. Cells were sedimented by centrifugation for 15 min at 3100g at 4 ^oC, the supernatant was removed and the cell pellets were washed twice with 0.9% NaCl. Cells were resuspended in supplemented M9 medium containing either: (a) 250 mg/L **2**, (b) 20 mg/L phe (**1**) (positive control), (c) no phe or analog (negative control). Protein expression was induced 10 min after the medium shift by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. Cells were cultured for 4 hours post-induction and protein expression was monitored by SDS polyacrylamide gel electrophoresis (PAGE, 12 %), using a normalized OD_{600} of 0.2 per sample.

Protein Purification

mDHFR as expressed in this work contains an N-terminal hexahistidine sequence, which was utilized to purify the protein by nickel affinity chromatography with stepwise pH gradient elution under denaturing conditions according to the recommendations of the supplier (Qiagen). The eluted protein was buffer-exchanged (Millipore, MWCO=5 kDa) against distilled water three times and the purified protein was subjected to matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis.

Tryptic Peptide Analysis

10 μl of purified protein in elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris, pH=4.5) was mixed with 90 μl 75 mM NH_4OAc, to which 2 μL of modified trypsin (Promega, 0.2 μg/μL) was added. The solution was allowed to digest overnight at room temperature. The reaction was quenched by addition of trifluoroacetic acid to pH < 4.0. The digest was subjected to sample clean-up by using a $ZipTip_{C18}$, which provided 2 μl of purified sample solution. 10 μl of the MALDI matrix (α-cyano-β-hydroxycinnamic acid, 10 mg/ml in 50% CH_3CN) was added, and 0.5 μl of the resulting solution was spotted directly onto the sample plate. Samples were analyzed in the linear mode on an Applied Biosystems Voyager DE Pro MALDI-TOF mass spectrometer.

Chemical Modification of Protein

Purified proteins (mDHFR-wt and mDHFR-2) were dissolved in 200 μl of PBS buffer (pH=6.0) and added to 20 μl of 5mM biotin hydrazide (BH, dissolved in PBS). Protein/BH mixtures were incubated at room temperature for 1 to 1.5 h. Reaction solutions were then washed twice with distilled water using a buffer-exchange column (Millipore, MWCO=5 kDa). Standard western blotting procedures were used to identify proteins modified with BH as well as those bearing an N-terminal hexahistidine tag.

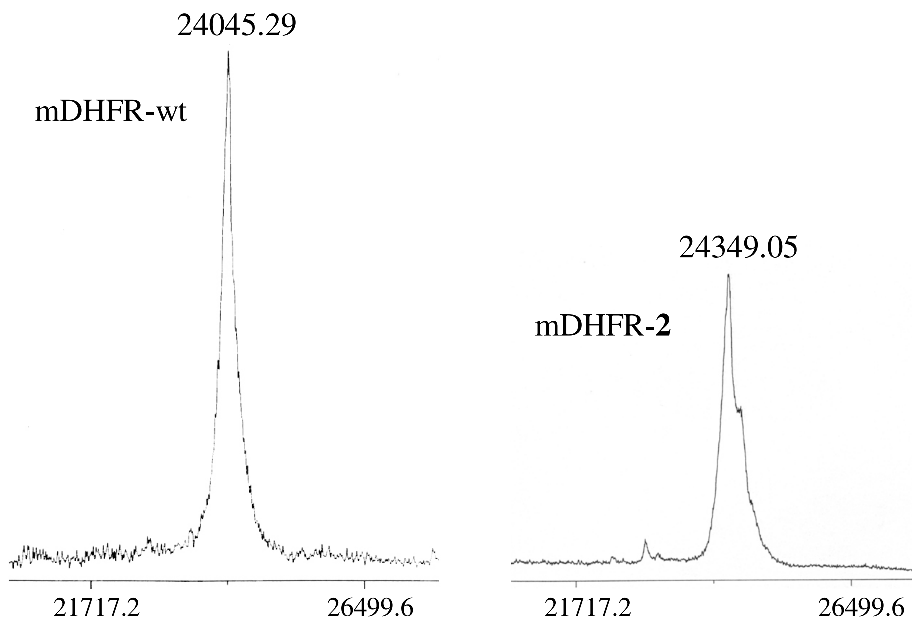

Figure 1: Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) of purified proteins.

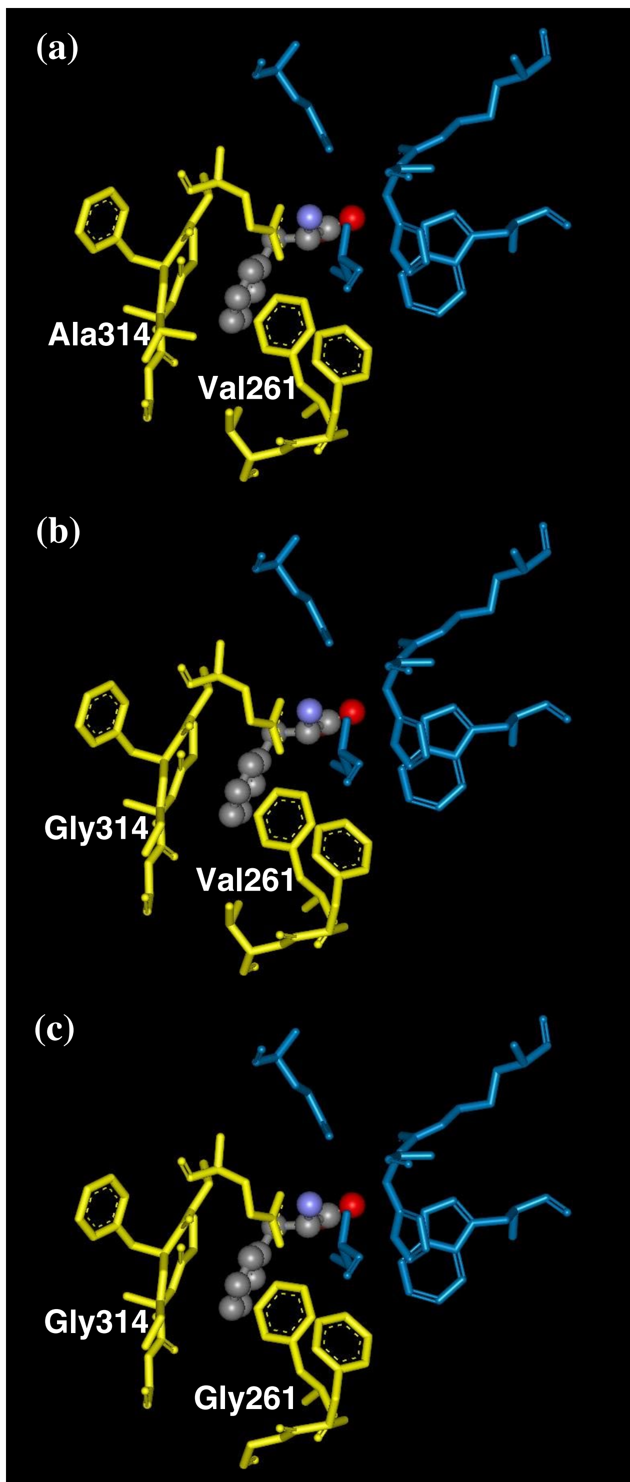

Figure 2: Active sites in *t*PheRS/Phe. Protein residues are shown as stick models and the substrate Phe is shown as a ball-and-stick model. Yellow protein residues are involved in hydrophobic interactions with the substrate. (a) wild type; (b) A314G mutant; (c) T261G/A314G mutant.