Supporting Information

for

Elongation Factor-P Modulates the Incorporation of Structurally Diverse Non-canonical Amino Acids into *E. coli* Dihydrofolate Reductase

Sasha M. Daskalova[a*], Larisa M. Dedkova[a], Rumit Maini[a], Poulami Talukder[a], Xiaoguang Bai[a], Sandipan Roy Chowdhury[a], Chao Zhang[a], Ryan C. Nangreave[a], and Sidney M. Hecht[a*]

[a]Biodesign Center for Bioenergetics, and School of Molecular Sciences, Arizona State University, Tempe, Arizona 85287, United States

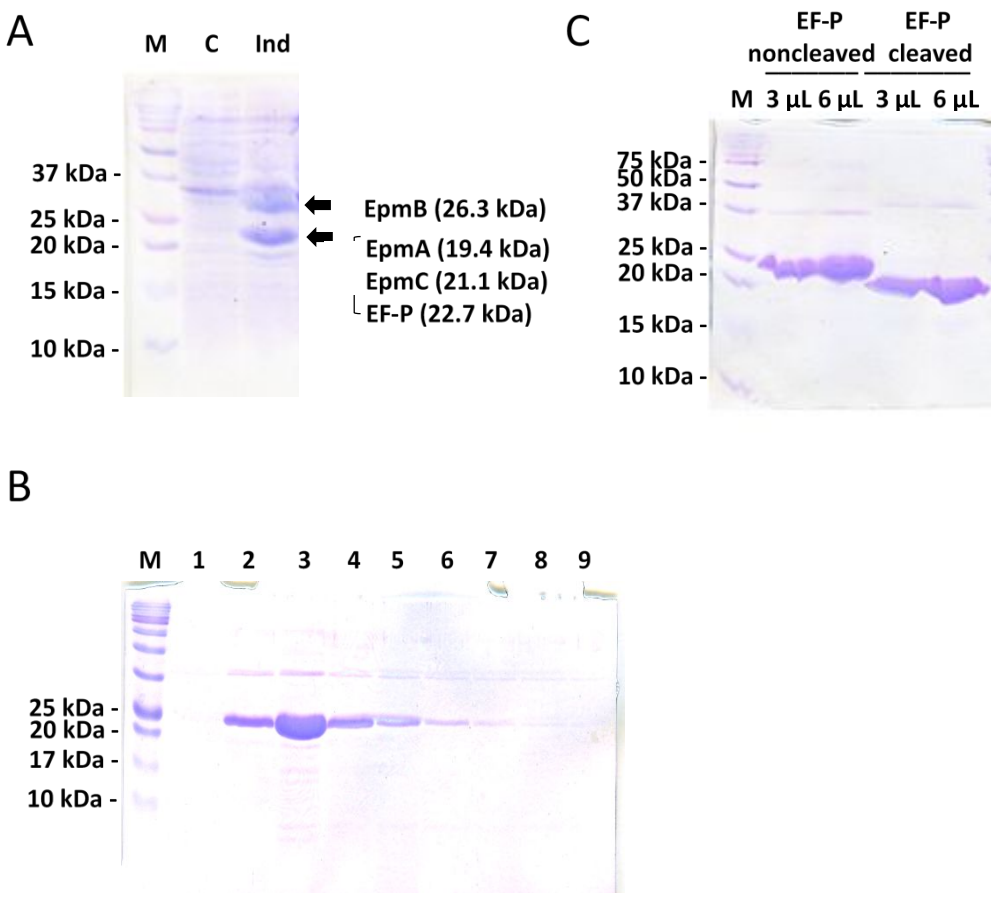

Figure S1. Expression and purification of recombinant post-translationally modified *E. coli* EF-P. (A) Co-expression of EF-P and its modifying enzymes following induction (Ind) with 1 mM IPTG. Arrows denote the positions of the induced proteins with their corresponding names listed to the right. (B) Purification of EF-P by immobilized metal affinity chromatography (IMAC). (C) Comparison between the electrophoretic mobility of EF-P before (left) and after (right) thrombin cleavage of the N-terminally positioned His-tag (1.9 kDa with the surrounding additional amino acids). All analyses were done by SDS-PAGE with Coomassie staining.

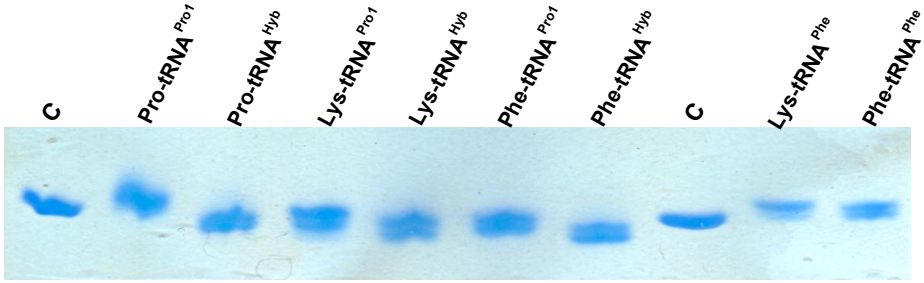

Figure S2. Efficiency of T4 RNA ligase mediated ligation between aminoacyl-pdCpA derivatives and $tRNA^{Pro1}$, $tRNA^{Hyb}$ and $tRNA^{Phe}$. The analysis was carried out by acidic polyacrylamide gel electrophoresis and methylene blue staining. Lanes C are control lanes with abbreviated tRNAs (tRNA-C_{OH}) not ligated with aminoacyl-pdCpA.

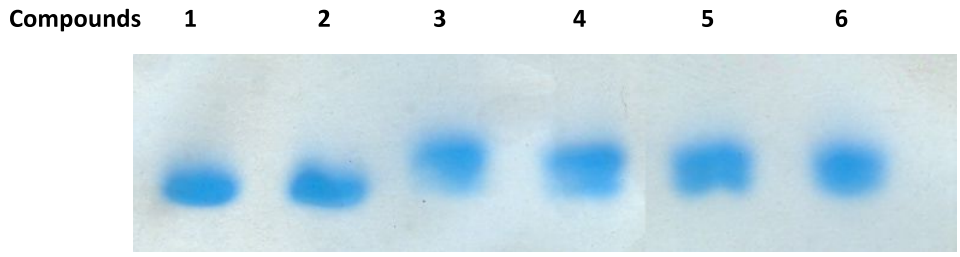

Figure S3. Efficiency of T4 RNA ligase mediated ligation between aminoacyl-pdCpA derivatives prepared from amino acid derivatives **1** – **6** and $tRNA^{Hyb}$-C_{OH}. The analysis was carried out by acidic polyacrylamide gel electrophoresis and methylene blue staining.

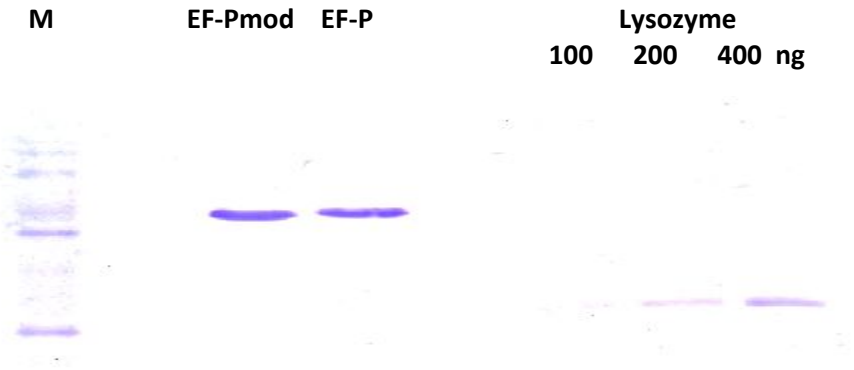

Figure S4. SDS-PAGE analysis of purified recombinant *E. coli* EF-P expressed in the absence (pETEF-P alone) or the presence of its modifying enzymes (pETEF-P/YjeA/YjeK/YfcM). Lysozyme quantity standards run alongside were used to estimate EF-P yields. M- molecular weight markers, 10, 15, 20, 25, 37 and 50 kDa. Expressed Strep-tagged EF-P alone is ~600 Da heavier compared to the cleaved post-translationally modified EF-P.

EXPERIMENTAL PROCEDURES

Chemical Synthesis. The syntheses of amino acid analogues **1**,[1] **2**,[2] **3**,[2] **4**,[3,4] **5**,[5], **6**[6] and **7**[3,7] were carried out as described in the original references. Each N-protected analogue was activated as its cyanomethyl ester and then used to form an aminoacyl-pdCpA ester. The aminoacyl-pdCpA esters were then ligated to $tRNA^{Hyb}$-C_{OH} via the agency of T4 RNA ligase in the presence of ATP.[8-11] The activated $tRNA^{Pro1}$ analogues were prepared in the same fashion.

Plasmid DNA Isolation Small-scale plasmid DNA isolation was performed using a GenElute Plasmid Miniprep Kit (Sigma). DNA templates for coupled *in vitro* transcription/translation were prepared using a E.Z.N.A. Plasmid DNA Maxi Kit (Omega Bio-tek) and additionally purified by extraction with 25:24:1 phenol-chloroform-isoamyl alcohol, pH 8.0, followed by concentration to 0.5-0.7 mg/mL by ethanol precipitation.

Site-directed Mutagenesis Site-directed mutagenesis was performed using a QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies). Template 1 (Ala9TAG10) was prepared using the wild type coding sequence of DHFR from pET28b:DHFR vector as a template and the following set of primers: forward 5′-GTCTGATTGCGGCGTTAGCCTAGGATCGCGTTATCGGC-3′/reverse 5′-GCCGATAACGCGATCCTAGGCTAACGCCGCAATCAGAC-3′. DHFR with Ala9Pro mutation was prepared using the wild-type coding sequence and the following set of primers: forward 5′-GTCTGATTGCGG CGTTACCGGTAGATCGCGTTATCGGC-3′/ reverse 5′-GCCGATAACGCGATCTACCG GTAACGCCGCAATCAGAC-3′. This was further mutated to introduce a Val10TAG mutation and prepare DHFR template 2 with the following set of primers:
forward 5′-GTCTGATTGCGGCGTTACCGTAGGATCGCGTTATCGGC-3′/
reverse 5′-GCCGATAACGCGATCCTACGGTAACGCCGCAATCAGAC-3′. In all occasions, following site-directed mutagenesis and *Dpn*I treatment, an aliquot of each reaction was transformed into XL10-Gold ultracompetent cells. Colonies were selected on LB50Kan plates. Several colonies were picked up, plasmid DNA was isolated and presence of the desired mutations was confirmed by sequencing.

Preparation of Runoff Expression Vectors for *E. coli* Suppressor $tRNA^{Pro1}$ and $tRNA^{Hyb}$. Sequences for both tRNAs, preceded by a T7 promoter along with the 5′ and 3′ flanking sequences[9] were prepared by *de novo* synthesis and subcloned into pUC18 vector between *EcoR*I and *Sph*I restriction sites. The vectors were transformed into DH5α competent cells and recombinant colonies were selected on LB agar plates, supplemented with ampicillin (100 μg/mL). Several colonies were picked up, their plasmid DNA was isolated and sequenced for verification.

Transcription and Purification of tRNA-C_{OH} Transcription of tRNA-C_{OH} was performed using *FokI*-digested runoff expression vectors coding for *E. coli* suppressor $tRNA^{Pro1}$ and $tRNA^{Hyb}$, or yeast $tRNA^{Phe}$, and Ampliscribe T7 Transcription Kit (Illumina). Synthesized tRNA-C_{OH} was purified by DEAE Sepharose CL-6B chromatography, via elution with a 0.1-0.7 M step gradient of NaCl in 0.1 M NaOAc buffer pH 5.0. Collected fractions were subjected to precipitation by

isopropanol and the pellets were redissolved in RNase-free water. An aliquot of each fraction was analyzed by acidic PAGE and methylene blue staining.

Ligation Between tRNA-C_{OH} and Pentenoyl-protected Aminoacyl-pdCpA
Ligation was performed at 37 °C for 1.5 h using 1 U/μL of T4 RNA ligase 1 (New England Biolabs), 1× ligation buffer (50 mM HEPES buffer, pH 7.5, and 15 mM $MgCl_2$), 1 μg/μL of tRNA-C_{OH}, 0.005 OD_{260}/μL of aminoacyl-pdCpA and 15% DMSO (v/v). The activated tRNA was precipitated with ethanol, resuspended in water, and deprotected using a final iodine concentration of 12.5 mM, and incubation at room temperature for 15 min. After another round of ethanol precipitation, each of the samples was dissolved in RNAse-free water to 3 μg/μL final concentration of aminoacyl-tRNA. To confirm the ligation, an aliquot was analyzed by acidic PAGE and methylene blue staining along with tRNA-C_{OH} as a negative control.

Preparation of *E. coli* S30 Extract A single, fresh colony of strain BL21(DE3) was inoculated into 3 mL of LB medium and grown for 3 h at 37 °C and 190 rpm. The bacterial suspension was used to inoculate 200 mL of LB medium. IPTG was added to a final concentration of 0.5 mM and the culture was grown at 37 °C and 190 rpm until OD_{600} reached 0.8-0.9. Cells were collected by centrifugation at 3500 × g for 15 min at 4 °C, washed three times with 20 mL/g of S30A buffer (0.014 M $Mg(OAc)_2$, 0.06 M KOAc, 0.01M TrisOAc pH 8.2, 0.01 M DTT, and 0.5 mL/L β-mercaptoethanol), once with 10 mL/g of S30B buffer (same as S30A buffer but with 0.05 mL/L β-mercaptoethanol), and then resuspended in 1.27 mL/g of S30C buffer (same as S30A buffer but without β-mercaptoethanol). For every milliliter of bacterial suspension, 0.3 mL of preincubation mix (0.32 M TrisOAc, pH 8.2, 9.38 mM $Mg(OAc)_2$, 13.4 mM ATP, 14.74 mM GTP, 84 mM phosphoenolpyruvate potassium salt, 4 mM DTT, and 0.048 mM amino acid mix) was added followed by the addition of 1 μL of pyruvate kinase (15 U/μL), 2 μL of 1 M $Mg(OAc)_2$ and 2 μL of lysozyme (50 mg/mL). Samples were incubated at 37 °C for 40 min, frozen at –80 °C overnight, thawed at 37 °C for 40 min, frozen again at –80 °C for 1 h and thawed at room temperature for 40 min. EGTA (0.1 M) was added to a final concentration of 2.5 mM followed by incubation at 37 °C for 30 min. $CaCl_2$ (0.1 M) was then added to a final concentration of 2.5 mM, and the samples were incubated at –80 °C for 1 h. Frozen cell lysates were centrifuged at 15000 × g for 1 h at 4 °C. Supernatants were carefully removed and stored in small aliquots at –80 °C until use.

Expression and Purification of Post-translationally Modified *E. coli* EF-P. *E. coli* expression vector pET28:EF-P/YjeA/YjeK/YfcM coding for *E. coli* EF-P and its three modified enzymes was obtained from Prof. Marina Rodnina and was employed for protein exprssion. The vector was transformed into DH5α competent cells and recombinant colonies were selected on LB agar plates, supplemented with kanamycin. Overexpression and purification of EF-P were carried out as described.[12,13]

Expression and Purification of Non-modified EF-P. Plasmid pETEF-P, having the wild-type EF-P gene only, was purchased from Synbio Technologies and transformed into BL- 21(DE-3) competent cells Recombinant colonies were selected on LB agar plates, supplemented with kanamycin. A single positive colony was transformed in LB medium (3 mL), supplemented with kanamycin and grown at 37 °C until ~1 OD_{600} was reached. The prepared culture was transferred into a flask containing 200 mL of LB medium, supplemented with the same antibiotic and

recombinant protein synthesis was initiated by the addition of IPTG to 0.5 mM concentration. The cells were centrifuged and resuspended in 2 mL of 0.1 M Tris-HCl buffer, pH 8.0, supplemented with 0.15 M NaCl and 1 mM EDTA. After lysozyme treatment and three freeze-thaw cycles, the lysate was centrifuged (15000 × g, 4 °C, 40 min) and the recombinant EF-P protein was purified by strep-tactin chromatography. The eluate was concentrated by Amicon centrifugal filter (10 kDa MW cutoff) and transferred into storage buffer (50 mM Tris-HCl, pH 7.4 containing 30% glycerol).

Coupled *in vitro* Transcription/translation Coupled *in vitro* transcription/translation for analytical purposes was performed in 10 µL reaction final volume, assembled by mixing 3.25 µL of 2.5× premix (87 mM Tris, 476 mM potassium glutamate, 75 mM ammonium acetate, 20 mM $Mg(OAc)_2 \bullet 4H_20$, 63 mM phosphoenol pyruvate potassium salt, 2 mM IPTG, 8.64% (w/v) PEG 8000, 0.2 mg/mL folinic acid, 2.5 mM cAMP, 1.25 mM UTP, 1.25 mM CTP, 5 mM ATP, 5 mM GTP, 5 mM DTT, 0.48 mg/mL of *E. coli* tRNA, pH 7.4), 0.1 µL 100× protease inhibitor cocktail (Roche), 0.2 µL of 100 mg/mL rifampicin, 3.25 µL of *E. coli* S-30 extract, 0.5 µL of 0.5 mM amino acid mix without methioni)ne, 0.5 µL of [^{35}S]Met (10.2 mCi/mL), 0.7 µL (500 ng) of plasmid DNA, 1.25 µL of aminoacylated or non-aminoacylated suppressor $tRNA_{CUA}$ (3 mg/mL), and 0.5 µL of EF-P (modified or non-modified, from stocks with different concentrations) or 0.5 µL of EF-P buffer (50 mM Tris HCl, pH 7.5, 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, 50% glycerol). All reactions were incubated for 1 h at 37 °C, quenched by addition of 2× SDS sample buffer (0.2 M Tris-HCl, pH 6.8, 0.2% SDS, 0.25% Orange G and 30% glycerol), and analyzed by SDS PAGE and autoradiography.

SDS-PAGE, Autoradiography and Staining SDS-PAGE was performed using homogenous polyacrylamide gels (4% stacking gel, 15% separating gel) prepared according to Laemmli' s protocol.[14] Protein samples were mixed with an equal volume of 2× loading dye (LiCor Biosciences) and incubated at 95 °C for 5 min. Aliquots (5-10 µL) were loaded onto the gel and run alongside Precision Plus Protein Dual Color Standards (BioRad). Following electrophoresis, gels employed for autoradiography were fixed with 40:10:50 ethanol-acetic acid-water for 1 h, then for an additional hour with 20:10:70 ethanol:acetic acid:water. The gels were then exposed overnight. Autoradiograms were scanned on Storm Scanner 820 (Amersham Bioscienes). Quantification was performed with Image Quant software Version 5.2. Alternatively, gels were stained overnight with Coomassie Brilliant Blue R 250 (0.25% (w/v) solution in 40:50:10 ethanol-acetic acid-water 40:10:50 and destained with 10% acetic acid. Images were scanned on a HP Scanjet 4370 using HP Solution software.

REFERENCES

(1) Talukder, P.; Dedkova, L. M.; Ellington, A. D.; Yakovchuk, P.; Lim, J.; E. V. Anslyn, E. V.; Hecht, S. M. Synthesis of Alanyl Nucleobase Amino Acids and Their Incorporation Into Proteins. *Bioorg. Med. Chem.* **2016**, *24*, 4177–4187.

(2) Maini, R.; Nguyen, D.; Chen, S.; Dedkova, L. M.; Roy Chowdhury, S.; Alcala Torano, R.; Hecht, S. M. Incorporation of β-Amino Acids into Dihydrofolate Reductase by Ribosomes Having Modifications in the Peptidyltransferase Center. *Bioorg. Med. Chem.* **2013**, *21*, 1088–1096.

(3) Zhang, C.; Bai, X.; Dedkova, L. M.; Hecht, S. M. Protein Synthesis with Conformationally Constrained Cyclic Dipeptides. *Bioorg. Med. Chem.* **2020**, *28*, 115780.

(4) Zhang, C.; Talukder, P.; Dedkova, L. M.; Hecht, S. M. Facilitated Synthesis of Proteins Containing Modified Dipeptides. *Bioorg. Med. Chem.* **2021**, *41*, 116210.

(5) Roy Chowdhury, S.; Chauhan, P. S.; Dedkova, L. M.; Bai, X.; Chen, S.; P. Talukder P.; Hecht, S. M. Synthesis and Evaluation of a Library of Fluorescent Dipeptidomimetic Analogues as Substrates for Modified Bacterial Ribosomes. *Biochemistry* **2016**, *55*, 2427–2440.

(6) Chen, S.; Maini, R.; Bai, X.; Nangreave, R. C.; Dedkova, L. M.; and Hecht, S. M. Incorporation of Phosphorylated Tyrosine into Proteins: *In Vitro* Translation and Study of Phosphorylated IκB-α and its Interaction with NF-κB. *J. Am. Chem. Soc.* **2017**, *139*, 14098–14108.

(7) Zhang, C.; Bai, X.; Dedkova, L. M.; Hecht, S. M. Local Conformational Constraint of Firefly Luciferase Can Affect the Energy of Bioluminescence and Enzyme Stability. *CCS Chemistry* **2022**, *4*, 1695–1707.

(8) Robertson, S. A.; Noren, C. J.; Anthony-Cahill, J.; Griffith, M. C.; Schultz, P. G. The Use of 5′-Phospho-2-deoxyribocytidylylriboadenosine as a Facile Route to Chemical Aminoacylation of tRNA. *Nucleic Acids Res.* **1989**, *17*, 9649–9660.

(9) Noren, C. J.; Anthony-Cahill, S. J.; Suich, D. J.; Noren, K. A.; Griffith, M. C.; Schultz, P. G. *In Vitro* Suppression of an Amber Mutation by a Chemically Aminoacylated Transfer RNA Prepared by Runoff Transcription. *Nucleic Acids Res*. **1989**, *18*, 83–89.

(10) Karginov, V. A.; Mamaev, S. V.; An, H.; Van Cleve, M. D.; Hecht, S. M.; Komatsoulis, G. A.; Abelson, J. N. Probing the Role of an Active Site Aspartic Acid in Dihydrofolate Reductase. *J. Am. Chem. Soc.* **1997**, *119*, 8166–8176.

(11) Dedkova, L. M.; Hecht, S. M. Expanding the Scope of Protein Synthesis Using Modified Bacterial Ribosomes, *J. Am. Chem. Soc.* **2019**, *141*, 6430–6447.

(12) Doerfel, L. K.; Wohlgemuth, I.; Kothe, C.; Peske, F.; Urlaub, H.; Rodnina, M. V. EF-P is Essential for Rapid Synthesis of Proteins Containing Consecutive Proline Residues. *Science* **2013**, *339*, 85–88.

(13) Katoh, T.; Wohlgemuth, I.; Nagano, M.; Rodnina, M. V.; Suga, H. Essential Structural Elements in $tRNA^{Pro}$ for EF-P-Mediated Alleviation of Translation Stalling. *Nat. Commun***.** **2016**, 7, 11657.

(14) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.