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Chemical shifts are reported in δ relative to tetramethylsilane for ^{1}H and ^{13}C spectra and relative to H_3PO_4 for ^{31}P spectra. Coupling constants (*J*) are reported in Hz. Chemical reagents were obtained from Sigma and Aldrich and used without further purification. Fast atom bombardment , chemical ionization, and electrospray mass spectra were obtained at the University of California, Irvine and University of California, Berkeley mass spectrometry laboratories. Elemental analyses were obtained at the University of California Berkeley microanalytical laboratory.

1-Methyl-2-Iodoterephthalate (6). 1-Methyl-2-aminoterephthalate (500 mg, 2.56 mmol) was added to a round-bottom flask charged with 5 ml of cold concentrated HCl. A solution of $NaNO_2$ (180 mg, 2.64 mmol) in 1 ml of H_2O was added dropwise, resulting in the evolution of a small amount of orange gas. The mixture was stirred for 30 min at room temperature and then filtered through glass wool into a solution of KI (4.30 g, 25.0 mmol) in 7 ml of H_2O. The dark red solution was stirred for 1 h and then diluted with CH_2Cl_2 (100 ml) and washed with saturated Na_2SO_3 (2 × 10 ml). The organic layer was washed with water (2 × 20 ml) and saturated NaCl (1 × 20 ml). The combined aqueous layers were back-extracted with CH_2Cl_2 (20 ml). The combined organic layers were dried over Na_2SO_4 and concentrated. The crude product was dissolved in a minimum amount of MeOH, and H_2O was added until the solution appeared slightly cloudy. Cooling to 4°C and subsequent filtration afforded 449 mg (57%) of a bright yellow solid, mp 155–157°C. IR (thin film): 2956, 2893, 2823, 2658, 2525, 1733, 1695, 1549 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.97 (s, 3H), 7.84 (d, 1H, *J* = 8.1), 8.12 (dd, 1H, *J* = 1.7, 8.1), 8.69 (d, 1H, *J* = 1.5). ^{13}C NMR (125 MHz, $CDCl_3$): δ 50.6, 129.4, 130.5, 132.3, 140.1, 142.5, 166.5, 168.6, 227.5. MS (chemical ionization) m/z 306 (M+). Anal. Calcd for $C_9H_7IO_4$: C, 35.32; H, 2.31; Found: C, 35.67; H, 2.21.

1-Methyl-2-Diphenylphosphinoterephthalate (7). Dry MeOH (3 ml), tetraethylammonium (0.3 ml, 2 mmol), compound **6** (300 mg, 1.00 mmol), and palladium acetate (2.2 mg, 0.010 mmol) were added to a flame-dried flask. The mixture was degassed *in vacuo*. While stirring under an atmosphere of Ar, diphenylphosphine (0.17 ml, 1.0 mmol) was added to the flask with a syringe. The resulting solution was heated at reflux overnight, and then allowed to cool to room temperature and concentrated. The residue was dissolved in 250 ml of a 1:1 mixture of CH_2Cl_2/H_2O and the layers were separated. The organic layer was washed with 1 M HCl (1 × 10 ml) and concentrated. The crude product was dissolved in a minimum amount of methanol and an equal amount of H_2O was added. The solution was cooled to 4°C for 2 h and the resulting solid was collected by filtration. The pure product was isolated as 245 mg (69%) of a golden yellow solid, mp 183–185°C. IR (thin film): 3051, 3001, 2950, 2608, 2481, 1720, 1587, 1562 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.75 (s, 3H), 7.28–7.35 (m, 11H), 7.63–7.67 (m, 1H), 8.04–8.07 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 52.4, 128.5, 128.6, 128.7, 129.0, 129.7, 130.6, 131.0, 131.8, 131.9, 133.7, 133.9, 135.4, 136.8, 152.7, 169.7, 221.9. ^{31}P NMR (160 MHz, $CDCl_3$): δ –3.67. MS (fast atom bombardment) *m*/*z* 365.1 (MH+). Anal. calculated for $C_{21}H1_7O_4P$: C, 69. 23; H, 4.70; Found: C, 69.24; H, 4.78.

Enzyme Purification and Activation Assays. The activation assay, which measures the amount of ^{32}P-radiolabeled ATP formed by the enzyme-catalyzed exchange of ^{32}P-pyrophosphate (PP_i) into ATP, was conducted in 150 μl of reaction buffer (pH 7.6, 20 mM imidazole/0.1 mM EDTA/10 mM β-mercaptoethanol/7 mM $MgCl_2$,/2 mM ATP/0.1 mg/ml BSA,/ 2 mM PP_i (in the form of sodium pyrophosphate) (NEN.) with a specific activity of 0.2–0.5 TBq/mole. Assays to determine whether the methionine analogs **2** or **3** support PP_i exchange in the presence of MetRS were conducted in solutions 75 nM in enzyme and 5 mM in the analog with a reaction time of 20 min. An additional assay to measure the amount of PP_i exchanged at 3.5 h in a reaction mixture that was 75 nM in enzyme and 15 mM in analog was also conducted for analog **2**. Quantitative kinetic parameters for analog **3** were obtained with enzyme concentrations of 50 nM and analog concentrations of 200 μM to 10 mM. Parameters for methionine were obtained by using concentrations ranging from 10 μM to 1 mM. Kinetic constants were calculated by nonlinear regression fit of the data to a Michaelis-Menten model.

Determination of Translational Activity. Buffers and media were prepared according to standard protocols. The *E. coli* methionine auxotroph CAG18491 (*λ*$^-$, *rph-1*, *metE3079*::*Tn10*), provided by the Yale *E. coli* Genetic Stock Center, was transformed with plasmids pREP4 and pQE15 (Qiagen, Chatsworth, CA), to obtain the conventional bacterial expression host CAG18491/pQE15/pREP4. The plasmid pQE15 encodes the protein mDHFR under the control of a bacteriophage T5 promoter. The expression plasmid also encodes an N-terminal hexahistidine sequence that permits purification of the target protein by immobilized metal chelate affinity chromatography. Furthermore, mDHFR contains eight methionine residues that can be replaced by methionine analogs, and its expression is easily monitored by SDS/PAGE) and visualized by Coomassie blue staining.

A modified bacterial host was prepared by transformation of strain CAG18491 with pREP4 and the expression plasmid pQE15-MRS (1) to yield CAG18491/pQE15-MRS/pREP4. In addition to encoding mDHFR, the expression plasmid pQE15-MRS carries the gene for MetRS under the control of the *metGp1* promoter. M9AA medium (50 ml) supplemented with 1 mM $MgSO_4$, 0.2 wt% glucose, 1 mg/liter thiamine chloride and the antibiotics ampicillin (200 mg/liter) and kanamycin (35 mg/liter) was inoculated with 2 ml of an overnight culture of either CAG18491/ pREP4/pQE15 or CAG18491/pQE15-MRS/pREP4. When the turbidity of the culture reached an OD_{600} of 0.8, the medium was exchanged to remove methionine. The cells were sedimented, and the cell pellet was washed twice with 20 ml of 1 × M9 salts. Cells were resuspended in 50 ml of the M9AA medium described above, without methionine. Test tubes containing 5-ml aliquots of the resulting culture were prepared and were supplemented with 500 mg/liter of either methionine, **2**, or **3**. A culture lacking methionine (or any analog) served as the negative control. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (Calbiochem) to a final concentration of 0.4 mM. Cultures were grown for 4.5 h, the OD_{600} was measured, and 1 ml of each of the samples was sedimented. After the supernatant was decanted, the cell pellets were resuspended in H_2O to a normalized OD_{600} of 10. Protein expression was monitored by SDS/PAGE and

visualized by Coomassie blue staining. The accumulation of the target protein mDHFR was taken as preliminary evidence for incorporation of the non-natural amino acid.

Protein Expression and Purification. Preparation of mDHFR from 1-liter cultures of CAG18491/pQE15/pREP4 supplemented with **3** was conducted by using the same procedures as those for determining translational activity of the methionine analogs. After the medium exchange, however, the cultures were resuspended in 1 liter of M9AA medium lacking methionine and supplemented with 400 mg/liter of **3**. Protein expression was induced as described above. These 1-liter cultures were sedimented after 4.5 h, and the cell pellet was stored at –80°C overnight.

mDHFR was purified from the cell pellet by using immobilized metal-affinity chromatography with stepwise pH gradient elution under denaturing conditions (Qiagen). Eluted protein was dialyzed batchwise against distilled water for 5 days. The dialysate was lyophilized to yield 35–40 mg of modified mDHFR from cultures grown on medium supplemented with 400 mg/liter of **3** (mDHFR-**3**), similar to the yield obtained for mDHFR from cultures supplemented with methionine (mDHFR-Met). Incorporation of **3** was confirmed by analyzing purified protein with amino acid analysis, N-terminal sequencing, or MALDI-MS. For mass spectral analysis, fragments of mDHFR-**3** were generated by trypsin digestion and analyzed by MALDI-MS from a matrix of α-cyano-4-hydroxycinnamic acid.

Protein Characterization. MS of the modified mDHFR both before and after ligation was prohibited by the fact that the N-terminal hexahistidine sequence was modified by *E. coli* during protein expression. The heterogeneous protein therefor appears as a range of molecular weights, which makes observing small changes in molecular weight impossible. However, tryptic digest of the protein provided fragments that could be examined by MALDI-MS as described in the text. The protein was further characterized by N-terminal sequencing and total amino acid analysis. The total amino acid analysis was carried out by Jack Presley (University of California, Davis).

1. Kiick, K. L., van Hest, J. C. M. & Tirrell, D. A. (2000) *Angew. Chem. Int. Ed. Engl.* **39,** 2148–2152.