Efficient Incorporation of Unsaturated Methionine Analogues into Proteins in Vivo

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Abstract: A set of eight methionine analogues was assayed for translational activity in *Escherichia coli*. Norvaline and norleucine, which are commercially available, were assayed along with 2-amino-5-hexenoic acid (**2**), 2-amino-5-hexynoic acid (**3**), *cis*-2-amino-4-hexenoic acid (**4**), *trans*-2-amino-4-hexenoic acid (**5**), 6,6,6-trifluoro-2-aminohexanoic acid (**6**), and 2-aminoheptanoic acid (**7**), each of which was prepared by alkylation of diethyl acetamidomalonate with the appropriate tosylate, followed by hydrolysis. The *E. coli* methionine auxotroph CAG18491, transformed with plasmids pREP4 and pQE15, was used as the expression host, and translational activity was assayed by determination of the capacity of the analogue to support synthesis of the test protein dihydrofolate reductase (DHFR) in the absence of added methionine. The importance of amino acid side chain length was illustrated by the fact that neither norvaline (**8**) nor **7** showed translational activity, in contrast to norleucine (**9**), which does support protein synthesis under the assay conditions. The internal alkene functions of **4** and **5** prevented incorporation of these analogues into test protein, and the fluorinated analogue **6** yielded no evidence of translational activity. The terminally unsaturated compounds **2** and **3**, however, proved to be excellent methionine surrogates: ^{1}H NMR spectroscopy, amino acid analysis, and N-terminal sequencing indicated ∼85% substitution of methionine by **2**, while **3** showed 90−100% replacement. Both analogues also function efficiently in the initiation step of protein synthesis, as shown by their near-quantitative occupancy of the N-terminal amino acid site in DHFR. Enzyme kinetics assays were conducted to determine the rate of activation of each of the methionine analogues by methionyl tRNA synthetase (MetRS); results of the in vitro assays corroborate the in vivo incorporation results, suggesting that success or failure of analogue incorporation in vivo is controlled by MetRS.

Introduction

There is growing interest in exploring and expanding the synthetic capacity of biological polymerization processes. An early—and critically important—example of such studies was the demonstration that "dideoxy" nucleotides can serve as substrates for DNA polymerases—an observation that underlies virtually all current DNA sequencing technology.[1] More recent investigations have shown the incorporation of modified or completely "synthetic" bases into nucleic acids,[2] while materials researchers have exploited the broad substrate range of the PHA synthases to prepare novel poly(β-hydroxyalkanoate)s (PHAs) with unusual physical properties.[3]

Among studies of this kind, attempts to expand the synthetic capacity of the translational apparatus may be uniquely challenging. The discriminatory power of the aminoacyl-tRNA synthetases, which safeguards the fidelity of protein biosynthesis in vivo, places severe limits on the set of amino acid structures that can be exploited in the engineering of natural and artificial proteins in vivo.

Several strategies for circumventing the specificity of the synthetases have been demonstrated. Chemical aminoacylation methods, introduced by Hecht and co-workers[4] and exploited by Schultz, Chamberlin, Dougherty, and others,[5] avoid the synthetases altogether, but (except for special cases) require the use of relatively inefficient cell-free translation protocols. Alteration of the synthetase activities of the cell is also possible, either through mutagenesis or through introduction of heterologous synthetases.[6,7] For some purposes, however, one can exploit the promiscuity of the wild-type synthetases, which have

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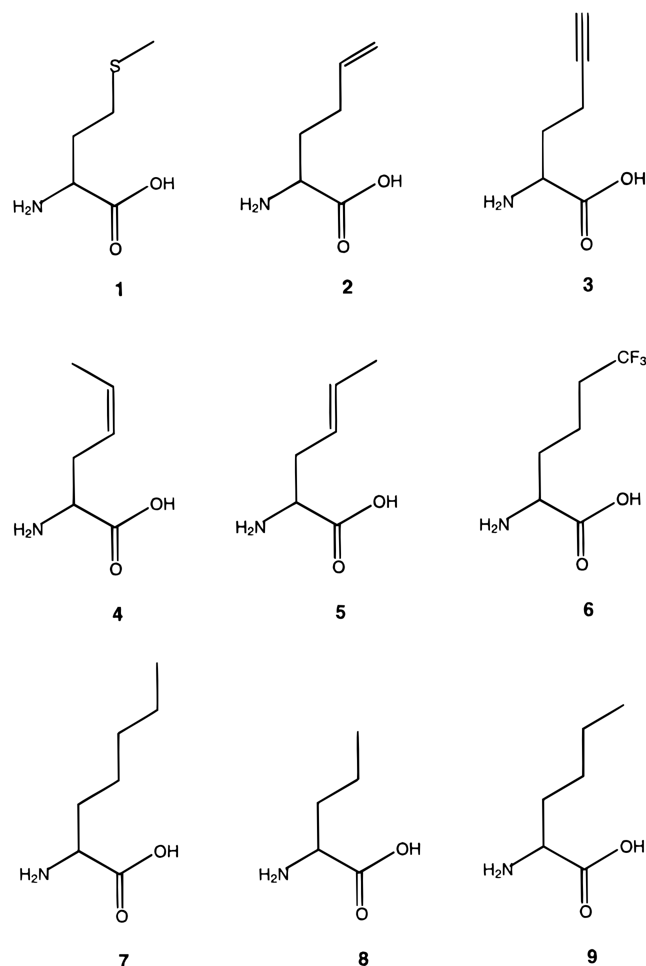
been shown to activate and charge substrates other than the canonical, proteinogenic amino acids.[8] This approach offers important advantages with respect to synthetic efficiency, in that neither chemical acylation of tRNA nor cell-free translation is required. The simplicity of the approach, and its capacity to provide relatively large quantities of engineered proteins, make this the method of choice whenever possible. We and others have demonstrated the capacity of the wild-type translational apparatus to utilize amino acid analogues bearing fluorinated,[9] unsaturated,[10] electroactive,[11] and other useful side chain functions; nevertheless, the number of amino acid analogues shown conclusively to exhibit translational activity in vivo is small, and the range of chemical functionality accessible via this route remains modest. These circumstances have prompted us to initiate a systematic search for new amino acid analogues that will allow the engineering of proteins with novel chemical and physical properties.

Several analogues of methionine (**1**) (specifically selenomethionine, telluromethionine, norleucine, trifluoromethionine, and ethionine[12]) have been shown to exhibit translational activity in bacterial hosts. These results turned our attention to the set of methionine analogues **2**−**8** (Chart 1), which we anticipated might allow the introduction of novel side chains into proteins made in vivo. We were particularly interested in the terminally unsaturated analogues **2** and **3**, because of the powerful and versatile chemistries characteristic of terminal alkenes and alkynes.[13] Homoallylglycine (Hag, **2**), for example, has been used to effect "covalent capture" of hydrogen-bonded peptide dimers via ruthenium-catalyzed olefin metathesis,[14] and the versatile chemistry of terminal alkynes suggests a variety of novel approaches to the derivatization of proteins outfitted with side chains derived from **3**. The conformationally restricted side chains of **4** and **5**, the trifluoromethyl group of **6**, and the varying steric and hydrophobic properties of **7** and **8**, are of interest with respect to the control of protein structure and stability.

Chart 1



We also examined the translational activity of norleucine (**9**), a close analogue of **7** and **8**, as a positive control for analogue incorporation. The results show definitely that **2** and **3** serve effectively as methionine surrogates in the initiation and elongation steps of protein biosynthesis in *Escherichia coli*. The remaining analogues **4**−**8** show no evidence of translational activity in the assays used to date.

Experimental Section

Synthesis of Amino Acid Analogues. Each of the analogues **2**−**7** was prepared by alkylation of diethyl acetamidomalonate with the appropriate alkyl tosylate followed by decarboxylation and deprotection of the amine function. This section provides information on general synthetic procedures and a detailed protocol for preparation of **2**. Similar methods were used to prepare **3**−**7**.

General Procedures. Glassware was dried at 150 °C and cooled under nitrogen prior to use. Tetrahydrofuran (THF) was freshly distilled from Na/benzophenone. *N*,*N*-Dimethylformamide (DMF) was distilled and stored over BaO. Pyridine (99.8%, anhydrous, Aldrich) and other reagents and solvents were used as received. ^{1}H NMR spectra were recorded on Bruker AC 200 and AMX 500 spectrometers and ^{13}C NMR spectra were recorded on a Bruker DPX 300 spectrometer. Column chromatography was performed with silica gel 60, 230−400 mesh (EM Science); silica 60-F254 (Riedel-de Haën) was used for thin-layer chromatography.

DL-2-amino-5-hexenoic acid (2).[15] **3-Buten-1-ol 4-methylbenzenesulfonate.** A solution of 3 g (42 mmol) of 3-buten-1-ol in 10 mL of dry pyridine was cooled in an ice bath. Tosyl chloride (7.9 g, 42

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mmol) was added. After 3 h of stirring, the mixture was poured into 30 mL of an ice/concentrated HCl 4/1 v/v solution, extracted with 60 mL of diethyl ether and dried overnight in the freezer over $MgSO_4$. The mixture was filtered, and the ether was evaporated to yield 7.22 g (76%) of 3-buten-1-ol 4-methylbenzenesulfonate as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 2.39−2.53 (m, 2H, J = 6.5 and 6.9 Hz, CH_2−CH═CH_2; and s, 3H, CH_3−Ar), 4.08 (t, J = 6.5 Hz, 2H, CH_2OSO$_2$), 5.09−5.15 (m, 2H, J_Z = 10.4, J_E = 16.6, J_{gem} = 3.1 Hz, CH_2−CH═CH_2), 5.57−5.82 (m, 1H, J_Z = 10.4, J_E = 16.6, J = 6.9 Hz, CH_2−C*H*═CH_2), 7.38 and 7.72 (d, 4H, J = 8.6 Hz, *Ar*).

Acetylamino-3-butenyl-propanedioic Acid Diethyl Ester. Diethyl acetamidomalonate, 1.56 g (6.9 mmol), was dissolved at room temperature under N_2 in 10 mL of dry THF. Potassium *tert*-butoxide (0.80 g, 7 mmol) was added under vigorous stirring. The mixture was heated for 2 h at 60 °C. 3-Buten-1-ol 4-methylbenzenesulfonate (1.5 g, 6.9 mmol) was added, and the mixture was heated under reflux for 2 days. The THF was removed, the residue was quenched with 10 mL of 1 M HCl, and the crude product was extracted with ethyl acetate (25 mL). The ethyl acetate solution was washed twice with 25 mL of water, dried over $MgSO_4$, filtered, and concentrated. The crude product was purified by column chromatography (eluent cyclohexane/ethyl acetate 2/1 v/v) to yield 0.82 g (44%) of acetylamino-3-butenyl-propanedioic acid diethyl ester. ^{1}H NMR ($CDCl_3$): δ 1.28 (t, 6H, J = 7.2 Hz, CH_3−CH_2), 1.78−2.0 (m, 2H, J = 8.3, 6.5 Hz, CH_2═CH−CH_2−CH_2), 2.08 (s, 3H, CONH−CH_3), 2.45 (m, 2H, J = 8.3 Hz, CH_2═CH−CH_2−CH_2), 4.25 (q, 4H, J = 7.2 Hz, CH_3−CH_2), 4.90−5.09 (m, 2H, J_Z = 10.4, J_E = 16.6, J_{gem} = 3.2 Hz, CH_2−CH═CH_2), 5.61−5.90 (m, 1H, J_Z = 10.4, J_E = 16.6, J = 6.5 Hz, CH_2−C*H*═CH_2), 6.78 (s, 1H, CON*H*−CH_3).

DL-2-Amino-5-hexenoic acid. The diethyl ester obtained as described above was hydrolyzed to the dicarboxylate by heating under reflux for 4 h in 25 mL of 10 w% NaOH. The solution was neutralized with 6 M HCl, and the solvent was evaporated. The diacid was extracted with 25 mL of methanol. After the solvent was evaporated, 20 mL of 1 M HCl was added and the solution was refluxed for 3 h. The solvent was evaporated, and the product was taken up in 10 mL of methanol. Propylene oxide (5 mL) was added, and the mixture was stirred overnight at room temperature. The precipitate was filtered and dried, yielding DL-2-amino-5-hexenoic acid (0.47 g, 63%). The product was recrystallized from EtOH/H_2O 2/1 v/v (0.28 g, 60%). The ^{1}H NMR data were in agreement with those of ref 16. ^{1}H NMR (D_2O): δ 1.78−2.0 (m, 2H, J = 6.4, 6.6 Hz, CH_2═CH−CH_2−CH_2), 2.08−2.20 (m, 2H, J = 6.1, 6.4 Hz, CH_2═CH−CH_2−CH_2), 3.75 (t, 1H, J = 6.1 Hz, H_2N−C*H*−COOH), 4.90−5.12 (m, 2H, J_Z = 10.5, J_E = 16.7, J_{gem} = 3.3 Hz, CH_2−CH═CH_2), 5.61−5.90 (m, 1H, J_Z = 10.5, J_E = 16.7, J = 6.6 Hz, CH_2−C*H*═CH_2). ^{13}C NMR (D_2O): δ 28.9 (CH_2═CH−*C*H_2−CH_2), 29.9 (CH_2═CH−CH_2−*C*H_2), 54.4 (H_2N−*C*H−COOH), 116.3 (CH_2−CH═*C*H_2), 137.3 (CH_2−*C*H═CH_2), 174.8 (*C*OOH).

Determination of Translational Activity. Buffers and media were prepared according to standard protocols.[17] The *E. coli* methionine auxotroph CAG18491 (λ^-, *rph-1, metE3079.:Tn10*) kindly provided by the Yale *E. coli* Genetic Stock Center, was transformed with plasmids pREP4 and pQE15 (Qiagen), to obtain the expression host CAG18491/pREP4/pQE15.

Protein Expression (5-mL Scale). M9AA medium (50 mL) supplemented with 1 mM $MgSO_4$, 0.2 wt % glucose, 1 mg/L thiamine chloride, and the antibiotics ampicillin (200 mg/L) and kanamycin (25 mg/L) was inoculated with 2 mL of an overnight culture of CAG18491/pREP4/pQE15. When the turbidity of the culture reached an optical density at 600 nm (OD_{600}) of 0.8, a medium shift was performed. The cells were sedimented for 10 min at 3030*g* at 4 °C, the supernatant was removed, and the cell pellet was washed twice with 20 mL of M9 medium. 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 200 μL of 1 mg/mL (0.27 mM) L-methionine (**1**) (positive control), DL-2-amino-5-hexenoic acid (**2**) (0.31 mM), DL-2-amino-5-hexynoic acid (**3**) (0.31 mM), *cis*- or *trans*-DL-2-amino-4-hexenoic acid (**4** or **5**) (0.31 mM), DL-6,6,6-trifluoro-2-amino hexanoic acid (**6**) (0.22 mM), DL-2-aminoheptanoic acid (**7**) (0.28 mM), L-norvaline (**8**) (0.34 mM), or L-norleucine (**9**) (0.31 mM), respectively. A culture lacking methionine (or any analogue) served as the negative control. Protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Samples were taken every hour for 4 h, the OD_{600} was measured, and the samples were sedimented. After the supernatant was decanted, the cell pellets were resuspended in 20 μL of distilled H_2O. Protein expression was monitored by SDS polyacrylamide gel electrophoresis (12% acrylamide running gel, 12 mA, 14 h), using a normalized OD_{600} of 0.2 per sample.

Protein Expression (1-L Scale). Similar procedures were used for preparation and isolation of DHFR from media supplemented with **1**, **2**, or **3**. The example presented is for medium supplemented with **3**. M9AA medium (100 mL) supplemented with 1 mM $MgSO_4$ 0.2 wt % glucose, 1 mg/L thiamine chloride, and the antibiotics ampicillin (200 mg/L) and kanamycin (25 mg/L) was inoculated with *E. coli* strain CAG18491/pREP4/pQE15 and grown overnight at 37 °C. This culture was used to inoculate 900 mL of M9AA medium supplemented as described. The cells were grown to an OD_{600} of 0.94 and the medium shift was performed as described for the small-scale experiments, followed by addition of 40 mL of 1 mg/mL DL-2-amino-5-hexynoic acid (**3**). IPTG (0.4 mM) was added, and samples were taken at 1-h intervals. OD_{600} was measured, the samples were sedimented and decanted, and the cell pellets were resuspended in 20 μL of distilled H_2O. Protein expression was monitored by SDS polyacrylamide gel electrophoresis (12% acrylamide running gel, 12 mA, 15 h).

Protein Purification. Approximately 4.5 h after induction, cells were sedimented (9800*g*, 10 min, 4 °C) and the supernatant was removed. The pellet was placed in the freezer overnight. The cells were thawed for 30 min at 37 °C, 30 mL of buffer (6 M guanidine-HCl, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8) was added, and the mixture was shaken at room temperature for 1 h. The cell debris was sedimented (15300*g*, 20 min, 4 °C), and the supernatant was subjected to immobilized metal affinity chromatography (Ni−NTA resin) according to the procedure described by Qiagen.[18] The supernatant was loaded on 10 mL of resin which was then washed with 50 mL of guanidine buffer followed by 25 mL of urea buffer (8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris, pH 8). Similar urea buffers were used for three successive 25-mL washes at pH values of 6.3, 5.9, and 4.5, respectively. Target protein was obtained in washes at pH 5.9 and 4.5. These washes were combined and dialyzed (Spectra/Por membrane 1, MWCO = 6−8 kDa) against running distilled water for 4 days, followed by batchwise dialysis against doubly distilled water for 1 day. The dialysate was lyophilized to yield 70 mg of modified DHFR (DHFR-Y). A similar procedure using medium supplemented with **2** yielded 8 mg of DHFR-E. A control experiment in 2× YT medium afforded 60 mg of DHFR. Amino acid analyses were provided by Dr. C. Cote, University of Massachusetts. Electrospray mass spectrometry was performed by Dr. W. McMurray, Yale University School of Medicine. N-terminal protein sequencing was done by Dr. J. Carlton, Louisiana State University.

Enzyme Purification and Activation Assays. The fully active, truncated form of methionyl tRNA synthetase (MetRS) was purified from overnight cultures of JM101 cells carrying the plasmid pGG3. (The plasmid, which encodes the truncated form of MetRS, was kindly donated by Professor Hieronim Jakubowski of UMDNJ−New Jersey Medical School, Newark, NJ.) The enzyme was purified by size exclusion chromotography as previously described.[19] Activation of methionine analogues by MetRS was assayed via the amino acid-dependent ATP-PP_i exchange reaction, also as previously described.[19,20] The assay, which measures the ^{32}P-radiolabeled ATP formed by the

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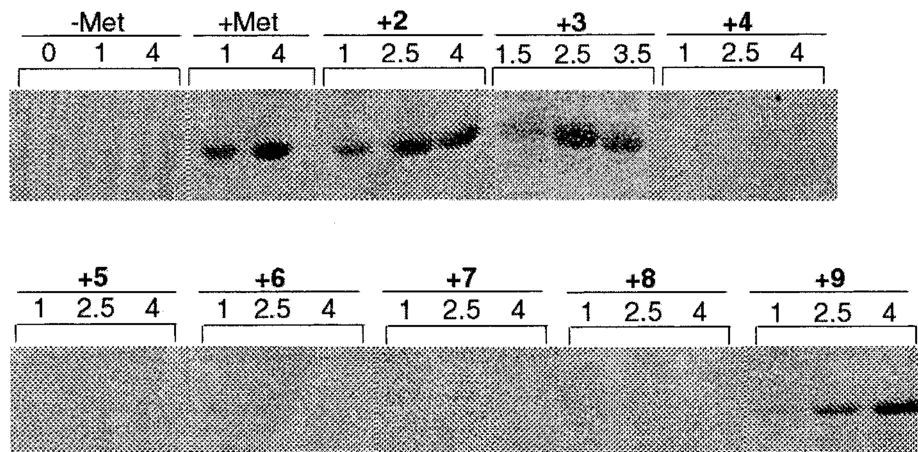

Figure 1. SDS−PAGE analysis of DHFR synthesis by *E. coli* strain CAG18491/pREP4/pQE15. Cultures were supplemented with methionine or with one of the analogues **2**−**9**, as indicated. Each lane is identified in terms of the time of analysis after addition of the inducer IPTG. DHFR is visualized by staining with Coomassie Brilliant Blue. The target protein can be detected only in cultures supplemented with methionine or with analogues **2**, **3**, or **9**, respectively.

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, and 2 mM PP_i (in the form of sodium pyrophosphate with a specific activity of approximately 0.5 TBq/mol)). Assays to determine whether the methionine analogues **2**−**9** are recognized by MetRS were conducted in solutions 50 nM in enzyme and 5 mM in the L-isomer of the analogue with a reaction time of 20 min. Kinetic parameters for analogue **5** were obtained with an enzyme concentration of 75 nM and analogue concentrations of 100 μM to 10 mM. Parameters for methionine were obtained by using concentrations ranging from 10 μM to 1 mM. K_m values for methionine matched those previously reported,[21] although the measured k_{cat} was somewhat lower than the literature value. Aliquots of 20 μL were removed from the reaction mixture at various time points and were quenched in 0.5 mL of a solution comprising 200 mM PP_i, 7% w/v $HClO_4$, and 3% w/v activated charcoal. The charcoal was rinsed twice with 0.5 mL of a 10 mM PP_i, 0.5% $HClO_4$ solution and was then resuspended in 0.5 mL of this solution and counted via liquid scintillation methods. Kinetic constants were calculated by nonlinear regression analysis.

Computation

Single-point energy ab initio calculations (Hartree−Fock model, 6-31G* basis set)[22] were performed for methionine and for analogues **2**, **3**, and **5** with fully extended side chains. Electron density maps are shown as surfaces of electron density 0.08 electrons/au^3. Isopotential plots are represented as surfaces where the energy of interaction between the amino acid and a point positive charge is equal to −10 kcal/mol. Calculations were performed by using the program MacSpartan (Wavefunction, Inc., Irvine, CA).

Results

Methionine Analogues. Methionine analogues **2**−**9** were investigated with respect to their capacity to support protein synthesis in *E. coli* cells depleted of methionine. Norvaline (**8**) and norleucine (**9**) are commercially available; the other analogues were prepared by alkylation of diethyl acetamidomalonate with the corresponding tosylates via standard procedures. In the cases of the *cis*- and *trans*-2-amino-4-hexenoic acids (**4** and **5**) the tosylates were prepared in situ, and because of fast exchange of the tosyl group with chloride ion, mixtures of the chloride and the tosylate were obtained. Hydrolysis of the malonate and conversion to the amino acid had to be performed under mild acidic conditions (see Experimental Section) for analogues **2**, **4**, and **5**; treatment with 6 N HCl, or reflux in 1 N HCl for more than 5 h led to HCl addition to the double bond. In all cases the analogues were obtained as racemates and were used as such.

Table 1. Protein Yield and Extent of Methionine Replacement

protein	yield (mg)[a]	replacement (%): amino acid analysis	sequencing	^{1}H NMR
DHFR-E	8	86	92	77
DHFR-Y	70	100	88	*b*

[a] Yield of purified protein obtained from 1 L of CAG18491/pREP4/pQE15 culture grown to OD_{600} = 0.94 prior to induction by addition of IPTG. The yield of DHFR obtained from control cultures supplemented with methionine was ca. 70 mg/L. [b] Not determined.

Protein Expression. *E. coli* strain CAG18491/pREP4/pQE15, which produces the test protein DHFR upon induction with IPTG, was used as the expression host. The parent strain CAG18491 is dependent on methionine for growth, owing to insertion of transposon *Tn10* into the *metE* gene, which is essential for the final step in the endogenous synthesis of methionine. Cultures were grown in minimal medium supplemented with methionine until a cell density corresponding to OD_{600} 0.8−1.0 was reached. Cells were sedimented, washed, and resuspended in minimal medium without methionine. Aliquots of the culture were then supplemented with one of the analogues **2**−**9**. Protein synthesis was induced with IPTG and cell growth and protein expression were followed over a 4-h period. Expression results are presented in Figure 1, and show clearly that analogues **2** and **3** exhibit translational activity sufficient to allow protein synthesis in the absence of methionine. Analogues **4**−**8** are not active in the assay reported here, while the known translational activity of norleucine (**9**) is confirmed. CAG18491/pREP4/pQE15 cultures did not grow in minimal media in which methionine was replaced by **2** and **3** at the time of inoculation.

Analysis of Protein Structure. The extent of replacement of methionine by analogues **2** and **3** was determined via amino acid analysis, N-terminal sequencing, and (for **2**) ^{1}H nuclear magnetic resonance spectroscopy (Table 1). Proteins containing **2** and **3** were designated DHFR-E (alk*e*ne) and DHFR-Y (alk*y*ne), respectively.

DHFR-E. Amino acid analysis of DHFR-E showed a methionine content of 0.5 mol % vs the value of 3.8 mol %

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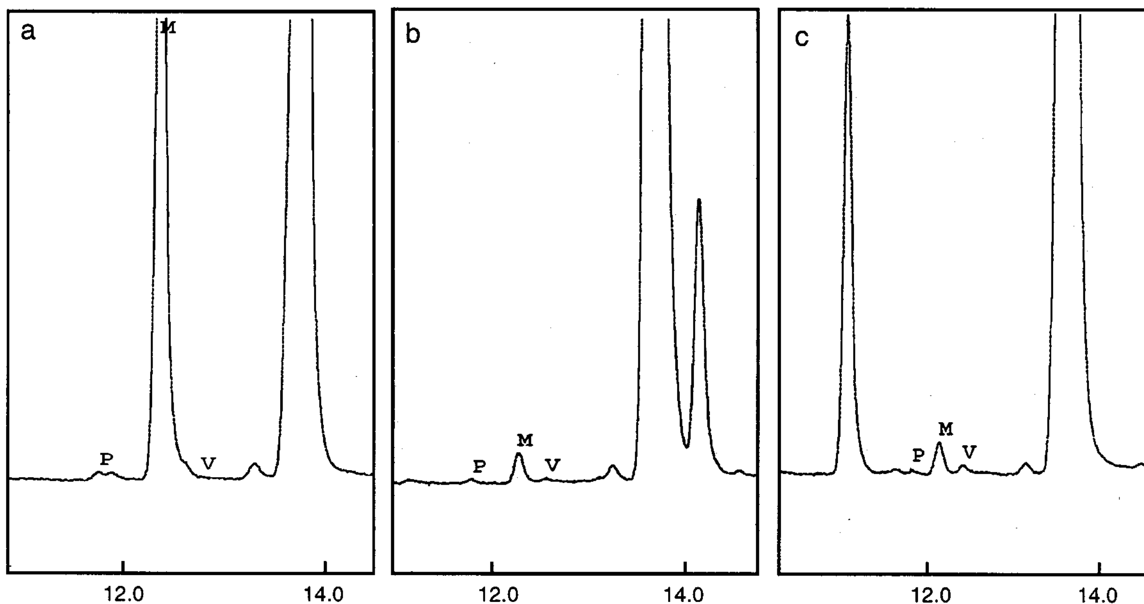

Figure 2. Determination of the occupancy of the initiator site in (a) DHFR, (b) DHFR-E, and (c) DHFR-Y. Chromatograms are shown for analysis of the N-terminal residue in each of the three proteins, as determined via Edman degradation. The signals corresponding to methionine, **2** and **3** elute at 12.3, 14.3, and 11.0 min, respectively. The strong signal at 13.8 min is due to piperidylphenylthiourea, a byproduct of the analysis. Signals assigned to **2** and **3** were verified by analysis of authentic samples of the analogues.

expected for DHFR. Although **2** appears to be unstable under the conditions used to hydrolyze the protein for amino acid analysis, assumption that the decrement in methionine content is due to replacement by **2** affords an estimate of 86% substitution by the analogue. This estimate is consistent with the results of N-terminal sequencing of DHFR-E (Figure 2), which indicates 92% occupancy of the initiator site by **2**. In the chromatograms shown in Figure 2, the signal due to methionine appears at a retention time of 12.3 min, while that from **2** elutes at 14.3 min. The retention time of the signal arising from **2** was verified by analysis of an authentic sample of the analogue. Retention of the N-terminal residue in DHFR was expected on the basis of the known correlation between the extent of methionine excision from *E. coli* proteins and the identity of the penultimate amino acid residue.[23] Finally, direct evidence for incorporation of the alkene function of **2** was obtained from ^{1}H NMR spectroscopy. The vinyl CH resonance of **2** appears at a chemical shift of 5.7 ppm in the spectrum of DHFR-E, and can be integrated to yield an estimate of 77% replacement of methionine by the unsaturated analogue. A yield of 8 mg of DHFR-E was obtained from a 1-L culture of CAG18491/pREP4/pQE-15 grown in M9AA medium supplemented with **2**, compared with 70 mg obtained from a similar experiment in medium supplemented with methionine.

DHFR-Y. Methionine could not be detected via amino acid analysis of DHFR-Y, suggesting quantitative replacement of methionine by the alkyne analogue **3**. N-terminal sequencing (Figure 2) indicated 88% occupancy of the initiator site by **3**. ^{1}H NMR analysis of DHFR-Y was consistent with near-quantitative replacement of methionine, as the thiomethyl resonance at 2.05 ppm—which is prominent in the spectrum of DHFR—could not be detected. New signals at 2.2−2.3 ppm—which are not observed in the spectrum of DHFR and which correspond to signals due to the β- and ϵ-protons of **3**—appeared in the spectrum of DHFR-Y, but were not integrated carefully owing to overlap with neighboring resonances. The yield of DHFR-Y obtained from M9AA medium supplemented with **3** was essentially identical to that of DHFR isolated from media supplemented with methionine.

(23) Hirel, P. H.; Schmitter, J. M.; Dessen, P.; Fayat, G.; Blanquet, S. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 8247.

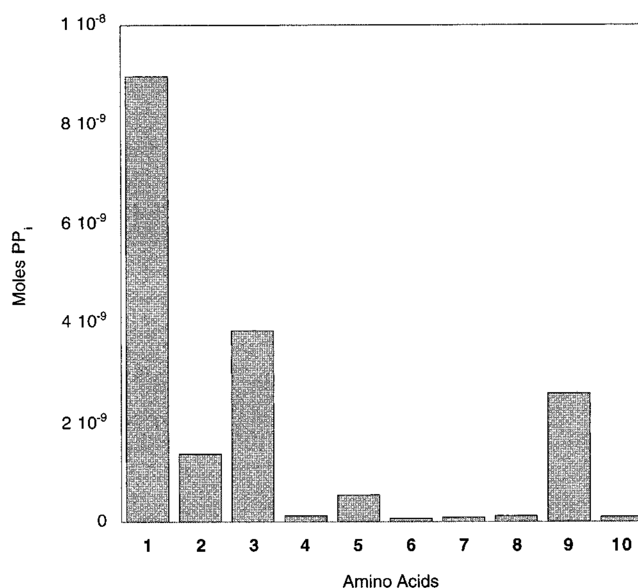

Figure 3. Activation of methionine and methionine analogues by MetRS. The amount of PP_i exchanged in 20 min is shown for methionine (**1**) and for methionine analogues **2**−**9**. The background (**10**) is given for a reaction mixture lacking both enzyme and amino acid.

Enzyme Assays. The relative rates of activation of methionine and methionine analogues **2**−**9** by MetRS were estimated by the ATP−PP_i exchange assay. The results shown in Figure 3 illustrate the amount of PP_i exchanged at a reaction time of 20 min under standard assay conditions (see Experimental Section). Methionine (**1**) is activated most efficiently by the enzyme, causing exchange of 9 nmol of PP_i over the time course of the reaction. Analogues **2** and **3** cause exchange of PP_i at rates similar to that of norleucine (**9**), while the remaining analogues **4** and **6**−**8** cause exchange of PP_i at levels no higher than background (Figure 3, lane 10). Although analogue **5** effects very slow exchange of PP_i, the activation rate is apparently too

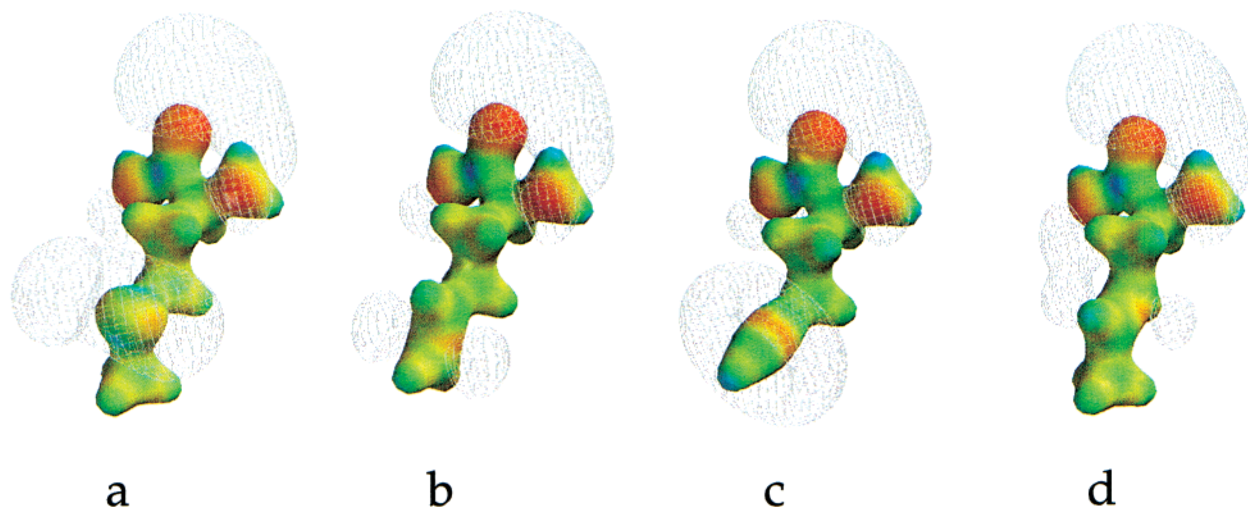

Figure 4. Electron density maps (colored surfaces) and negative isopotential surfaces (meshes) for methionine (a) and for analogues **2**, **3**, and **5** (b−d, respectively). The electron density maps indicate electron-rich (red) and electron-poor (blue) regions of each molecule. For simplicity, the amino acid form is shown; this avoids representation of the highly extended isopotential surface of the carboxylate anion of the zwitterion and facilitates comparison of side-chain electronic structure.

low to support protein synthesis at a level that is detectable in our in vivo assays. Kinetic parameters were determined for methionine and **5** as outlined in the Experimental Section. Comparison of the k_{cat}/K_m values obtained for methionine (0.55 s^{-1} μM^{-1}) and **5** (1.2×10^{-4} s^{-1} μM^{-1}) show that **5** is activated 4600-fold less efficiently than methionine by MetRS.

Discussion

A bacterial host strain (designated CAG18491/pREP4/pQE15) suitable for testing the translational activity of methionine analogues **2**−**8** was prepared by transformation of *E. coli* strain CAG18491, a methionine auxotroph, with the repressor plasmid pREP4 and the expression plasmid pQE15. pQE15 encodes mouse dihydrofolate reductase (DHFR) under control of a bacteriophage T5 promoter, and appends to DHFR an N-terminal hexahistidine sequence that facilitates purification of the protein by immobilized metal affinity chromatography. DHFR contains eight methionine residues, each a potential site for substitution by analogues **2**−**8**. The translational activity of each analogue was assayed on the basis of its capacity to support synthesis of DHFR in cultures of CAG18491/pREP4/pQE15 that had been depleted of methionine. In those instances in which the test protein was detected by gel electrophoresis (i.e., for **2** and **3**), the modified DHFR was purified and analyzed to determine the extent of methionine replacement by the analogue.

The results of the in vivo assays illustrated in Figure 1 show clearly that homoallylglycine (**2**) and homopropargylglycine (**3**) serve effectively as methionine surrogates in bacterial protein synthesis. In contrast, analogues **4**−**8** do not support measurable levels of protein synthesis in bacterial cultures depleted of methionine. It is highly unlikely that recognition by the elongation factors of the ribosome or transport into the cell are the limiting factors for incorporation of these analogues. The ribosome is remarkably permissive toward amino acid analogues with widely varying chemical functionality, as has been demonstrated by the numerous analogues incorporated into proteins in in vitro translation experiments.[5] Transport of analogues **4**−**8** into the cell is indicated by a number of literature reports. Analogue **4** is an antagonist for methionine, inhibiting the growth of *E. coli* cells;[24] **5** has been incorporated into proteins in *E. coli* cells with appropriately engineered MetRS activity;[25] and **8** replaces leucine in human hemoglobin expressed in *E. coli*.[26] Although there is no similar evidence reported for analogues **6** and **7**, the fact that trifluoromethionine and ethionine are incorporated into proteins expressed in *E. coli*[12] suggests that neither the trifluoromethyl group nor the longer side chain will inhibit transport of analogues **6** and **7** into *E. coli* cells.

The results of the in vitro enzyme assays shown in Figure 3 are consistent with the in vivo results, as the analogues that support the highest rates of PP_i exchange also support protein synthesis in the absence of methionine. Although the in vitro results indicate that **5** is recognized by MetRS, comparison of the k_{cat}/K_m values of methionine and **5** demonstrate that **5** is activated 4500-fold less efficiently than methionine; thus it is not surprising that **5** does not support measurable protein synthesis in our in vivo experiments. Consideration of the in vivo and in vitro results, along with the reports cited earlier, suggests that transport is not limiting and that analogue incorporation is controlled by the MetRS.

Although the crystal structure of an active tryptic fragment of the *E. coli* MetRS (complexed with ATP) has been reported,[27] the corresponding structure with bound methionine is not yet available. Inferences concerning the mechanism of methionine (or analogue) recognition by MetRS must therefore be made with care, and have heretofore been made indirectly, on the basis of sequence comparison and site-directed mutagenesis.[21,28] The following discussion is therefore speculative.

Figure 4 compares the equipotential surfaces calculated for methionine and for analogues **2**, **3** and **5**. That **2** might serve as a substrate for the methionyl-tRNA synthetase is not surprising, given the similar geometries accessible to **1** and **2**, the availability of π-electrons near the side-chain terminus of **2**, and the known translational activity of norcleucine (**9**), the saturated analogue of **2**. The high translational activity observed

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for **3**, on the other hand (i.e., near-quantitative replacement of methionine without loss of protein yield), was not anticipated, since the collinearity of side-chain carbons 4−6 imposes on **3** a geometry substantially different from that of methionine. On the other hand, the electron density associated with the triple bond of **3** is positioned similarly to that of the thioether of the natural substrate, despite the differences in side-chain geometry. Furthermore, given the important roles assigned to residues Phe197 and Trp305 in the *E. coli* methionyl-tRNA synthetase,[21,28] alkynyl C−H/π contacts[29] and the polarizability of the unsaturated side chain may also play significant roles in recognition of **3** by the enzyme. Figure 4 also compares the geometries of **1** and **5**, the latter an analogue neither activated efficiently by the MetRS in vitro nor measurably incorporated into protein in vivo. Although the geometries of **1** and **5** appear similar in the representation shown, the fixed planarity of the C_4−C_5 bond may preclude the side-chain conformation required for efficient activation of **5** by MetRS. Appropriate engineering of the MetRS activities of *E. coli* imparts translational activity to **5**,[25] and efforts are underway to permit recognition of additional methionine analogues by the MetRS.

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