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Protein Engineering by In Vivo Incorporation of Non-Natural Amino Acids: Control of Incorporation of Methionine Analogues by Methionyl-tRNA Synthetase

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Abstract—The incorporation of non-natural amino acids is an important strategy for engineering novel chemical and physical properties into natural and artificial proteins. The incorporation of amino acids into proteins in vivo is controlled in large part by the aminoacyl-tRNA synthetases (AARS). We have measured kinetic constants for in vitro activation of a set of methionine analogues by methionyl-tRNA synthetase (MetRS) via the ATP–PP_i exchange reaction. Activation of methionine analogues in vitro correlates well with the ability of these analogues to support protein synthesis in vivo, substantiating the critical role of the AARS in controlling the incorporation of non-natural amino acids into proteins. Methionine analogues with k_{cat}/K_m values 2000-fold lower than those for methionine can support synthesis of a typical target protein (mDHFR) under standard conditions of protein expression. The kinetic constants correlate well with observed protein yields from a conventional bacterial expression host, indicating that the MetRS activity of the host can control the level of protein synthesis under certain conditions. Furthermore, increasing the MetRS activity of the bacterial host results in increased protein synthesis in media supplemented with the methionine analogues homoallylglycine and norleucine. These results suggest new strategies for incorporation of non-natural amino acids via manipulation of the AARS activity of a bacterial host.

Introduction

Although many advances in synthetic polymer chemistry have been made over the last several decades to provide the polymer chemist with increasing control over the structure of macromolecules,[1–7] none have provided the level of control that is the basis of the exquisite catalytic, informational, and signal transduction capabilities of proteins and nucleic acids.[8] For this reason, we have been investigating the design and bacterial synthesis of artificial proteins that exhibit novel and potentially useful materials properties. Harnessing the molecular weight and sequence control provided by in vivo synthesis should permit control of folding, functional group placement, and self assembly at the angstrom length scale. Indeed, proteins produced by this method exhibit predictable chain-folded lamellar architectures,[9–12] unique smectic liquid-crystalline structures with precise layer spacings,[13] and controlled reversible gelation.[14] The demonstrated ability of these protein polymers to form unique macromolecular architectures will be of certain importance for engineering materials with interesting liquid-crystalline, crystalline, surface, electronic, and optical properties. The novel chemical and physical properties that can be engineered into protein polymers may be expanded by the precise placement of non-natural amino acids.

We have focused on in vivo incorporation of non-natural amino acids into proteins owing to the synthetic advantages it offers with respect to other methods for analogue incorporation. Introduction of non-natural amino acids can be achieved relatively simply via solid-state peptide synthesis. While this method circumvents all biosynthetic machinery, the multistep procedure is limited to synthesis of peptides less than or equal to approximately 50 amino acids in length and is therefore not suitable for producing protein materials. Chemical aminoacylation methods, introduced by Hecht and coworkers[15] and exploited by Schultz, Chamberlin, Dougherty, and others[16–19] provide a powerful method for the site-specific incorporation of non-natural amino acids. But because these methods (except in special cases) require the use of cell-free translation protocols that limit protein yields, they are also unsuitable for production of protein materials. The simplicity of the in vivo approach, its relatively high synthetic efficiency, and its capacity for multisite substitution make it the method of choice for production of protein materials whenever possible.

The capacity for multisite substitution is an attractive feature of protein engineering with non-natural amino acids, as such substitution can cause important changes in

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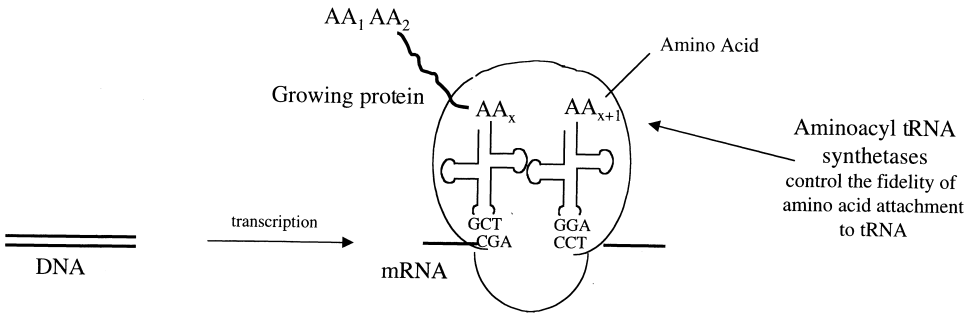
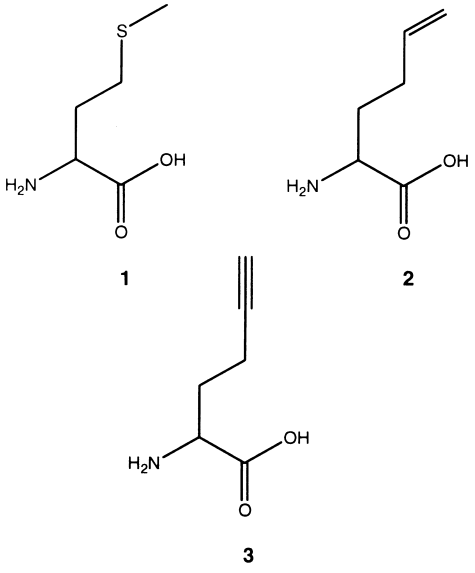


Figure 1. Schematic diagram of in vivo protein synthesis. The DNA message is translated into an amino acid sequence via the pairing of the codon of the messenger RNA (mRNA) with the complementary anticodon of the aminoacyl-tRNA. Aminoacyl-tRNA synthetases control the fidelity of amino acid attachment to the tRNA.

protein behavior. For example, incorporation of selenomethionine in place of methionine has long been known to facilitate protein structure determination by X-ray crystallography.[20] The incorporation of fluorinated functional groups into proteins has imparted to protein films the low surface energy characteristic of fluoropolymers; contact angles of hexadecane on fluorinated protein polymers (70°) are much higher than those on unfluorinated controls (17°).[21] Incorporation of trifluoroleucine in place of leucine also results in increases in the thermal stability of leucine zipper peptides;[22] these results may have important consequences for increasing protein stability, improving protein assembly, or strengthening ligand-receptor interactions. Furthermore, alkene functionality introduced into artificial proteins via dehydroproline can be quantitatively modified via bromination and hydroxylation.[23] The chemistries of the above functional groups are distinct from the chemistries of the amine, hydroxyl, thiol, and carboxylic acid functional groups characteristic of proteins; this makes their incorporation particularly attractive for targeted chemical modification of proteins.

The in vivo incorporation of non-natural amino acids into proteins is controlled most stringently by the aminoacyl-tRNA synthetases (AARS), the class of enzymes that safeguards the fidelity of amino acid incorporation into proteins (Fig. 1). We and others have demonstrated the ability of the wild-type translational apparatus to use non-natural amino acids with fluorinated,[21,24] unsaturated,[23,25,26] electroactive,[27] and other side chain functions.[28–31] Nevertheless, the number of amino acids shown conclusively to exhibit translational activity in vivo is small, and the chemical functionality that has been accessed by this method remains modest. Our most recent efforts have therefore been directed toward understanding the recognition of amino acid analogues by the AARS in order to expand the novel chemical and physical properties that can be engineered into proteins in vivo.

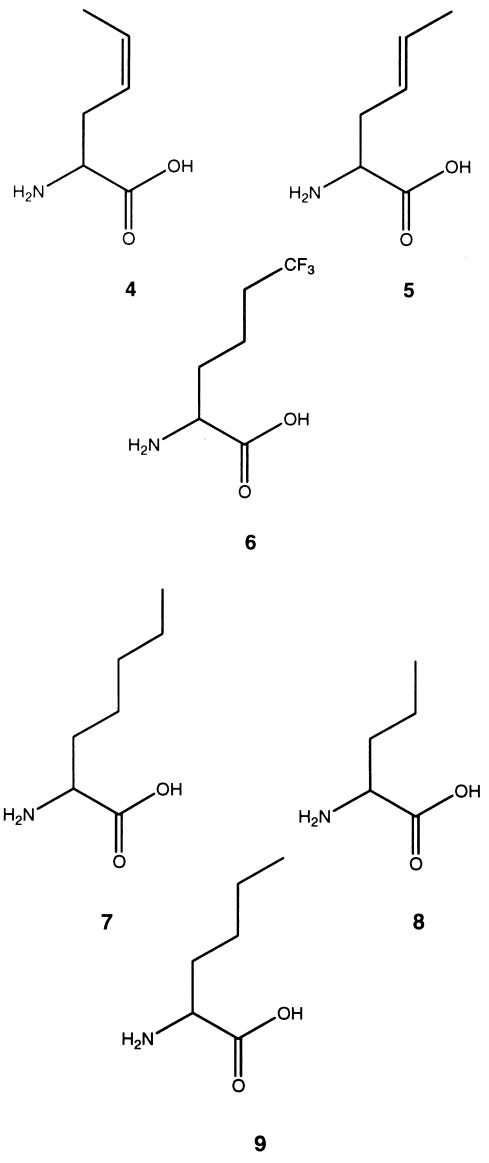
In recent studies of methionine analogues **2**–**9**,[26] we demonstrated that **2** and **3** can be incorporated into proteins with extents of substitution up to 98%. (The incorporation of **9** had been previously reported).[30] In contrast, **4**–**8** do not support protein synthesis in the absence of methionine in a conventional bacterial expression host; investigation of the activation of the analogues by methionyl-tRNA synthetase (MetRS) indicated that **4**–**8** are not efficiently activated by the enzyme. Overproduction of MetRS in the bacterial host, however, permits incorporation of **5**, which shows very slow exchange of PP_i in in vitro activation assays.[32] Furthermore, equipping a bacterial host with a phenylalanyl-tRNA synthetase (PheRS) active site mutant (PheRSA294G) allows incorporation of *p*-Cl- and *p*-Br-phenylalanine (*p*-Cl-Phe and *p*-Br-Phe) into proteins in vivo.[33,34] These results indicate that the AARS are appropriate targets for studies aimed at the incorporation of non-natural amino acids into proteins in vivo, and they also suggest that neither transport into the cell nor recognition by the elongation factors or the ribosome limits the incorporation of these non-natural amino acids into proteins in vivo.



a) $AARS + aa + ATP \rightleftharpoons [AARS{:}aa{\sim}AMP] + PP_i$

b) $[AARS{:}aa{\sim}AMP] + tRNA^{aa} \longrightarrow aa{\sim}tRNA^{aa} + AMP + AARS$

Scheme 1. Activation (a) and aminoacylation (b) steps of amino acid attachment to tRNA.



In the study described here, the in vitro activation of **2**–**9** by MetRS has been characterized in order to determine the roles of the synthetase in controlling analogue incorporation and protein yield in media supplemented with non-natural amino acids. Our investigations of methionine are motivated by the importance of methionine in mediating protein structure and protein–protein recognition processes;[35–37] controlled incorporation of methionine analogues may permit purposeful manipulation of these phenomena. Furthermore, the analogues **2** and **3**, which replace methionine in vivo, should be useful for chemical modification of proteins by olefin metathesis,[38,39] palladium-catalyzed coupling,[40–42] and other chemistries.[43]

Results and Discussion

The attachment of an amino acid to its cognate tRNA proceeds in two steps (Scheme 1). Activation, the first step, involves the enzyme-catalyzed formation of an aminoacyl adenylate (designated *aa-AMP* in Scheme 1) and can be studied by monitoring the rate of exchange of radiolabeled pyrophosphate (^{32}P–PP_i) into ATP.[44] Aminoacylation, the second step, can be studied by monitoring the amount of radiolabeled amino acid attached to tRNA in the presence of the enzyme. Because initial recognition of an amino acid by its AARS is perhaps the most critical step in the incorporation of non-natural amino acids into proteins in vivo, we have focused on the in vitro activation of methionine analogues by MetRS and have compared the results to those obtained in studies of in vivo incorporation.

The rates of activation of **2**–**9** by MetRS were determined by the ATP–PP_i exchange assay, and were found to correlate well with the results of our in vivo studies; only analogues **2**, **3**, **5**, and **9** (those which had been shown to support protein synthesis) exhibit measurable exchange of PP_i.[26,32] The kinetic parameters k_{cat} and K_m were determined for each of these analogues; the results are summarized in Table 1. Our measured K_m for methionine matched previously reported values,[45] the value determined for k_{cat} was slightly lower than the literature value. Comparison of the k_{cat}/K_m values for each of the analogues with that for methionine shows that the analogues are 500-fold to 4700-fold poorer substrates for MetRS than methionine.

Table 1 also demonstrates that methionine analogues that are activated up to 2000-fold more slowly by MetRS than methionine can support protein synthesis in a conventional bacterial host in the absence of methionine. (Poorer substrates, such as **5**, require modification of the MetRS activity of the bacterial host in order to support protein synthesis.[32]) These results are comparable to those reported previously for the activation and in vivo incorporation of phenylalanine analogues;[46–48] comparisons for other amino acids have been limited by a lack of in vitro activation data. The data suggest that non-natural amino acids can support protein synthesis in vivo even with surprisingly inefficient activation of the amino acid by its AARS, and that activation of methionine analogues by MetRS does indeed govern their ability to support protein synthesis in vivo.

Based on these results, it seemed likely that the kinetics of

Table 1. Kinetic parameters for methionine analogues in the ATP–PP_i exchange reaction and protein yields for bacterial cultures supplemented with the analogues.

Analogue	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	Protein yield, mg/L
1	24.3±2	13.3±0.2	5.47×10^{-1}	35
3	2415±170	2.60±0.3	1.08×10^{-3}	35
9	4120±900	2.15±0.6	5.22×10^{-4}	20
2	4555±200	1.35±0.1	2.96×10^{-4}	10
5	15,675±250	1.82±0.6	1.16×10^{-4}	0

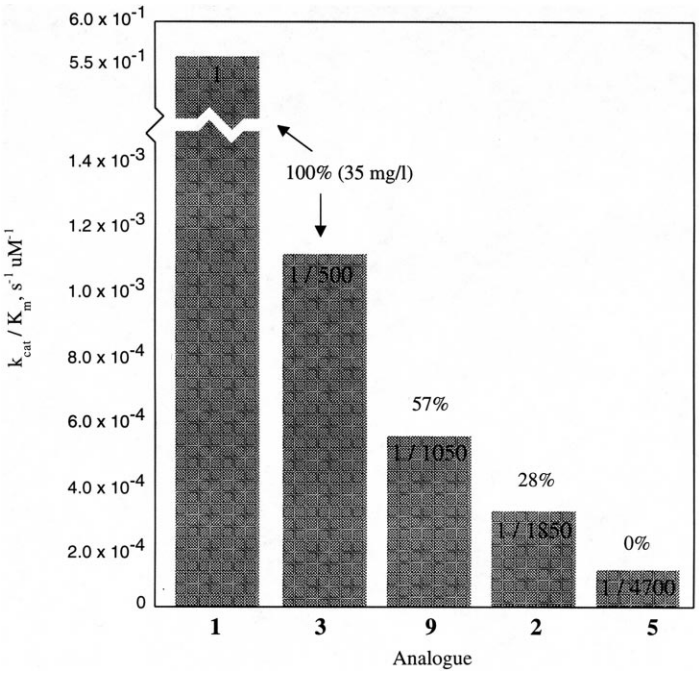


Figure 2. Comparison of the kinetic parameters for methionine analogues in the ATP–PP_i exchange reaction and relative protein yields from conventional bacterial host cultures supplemented with the analogues.

analogue activation would limit the rate and yield of protein synthesis in bacterial cultures supplemented with methionine analogues that are poor substrates for MetRS. We investigated this correlation by comparing the kinetic constants for analogue activation by MetRS with the yield of the target protein murine dihydrofolate reductase (mDHFR) obtained from 1-liter cultures of the bacterial host CAG18491/pQE15/pREP4. The bacterial host was produced by transforming the *E. coli* methionine auxotroph CAG18491 with the expression plasmid pQE15 and the repressor plasmid pREP4. The expression plasmid pQE15 encodes mDHFR under control of a bacteriophage T5 promoter and an N-terminal hexahistidine sequence that permits purification of the target protein by immobilized metal chelate affinity chromatography.[49]

The kinetic constants for analogue activation and the corresponding protein yields are listed in Table 1 and shown in Fig. 2. Analogues with the highest k_{cat}/K_m values also support the highest levels of protein synthesis; the protein yields scale remarkably well with k_{cat}/K_m, at least for the poorer substrates. Analogue **3** supports protein synthesis with yields equivalent to those obtained with methionine, despite the fact that **3** is a 500-fold poorer substrate for MetRS than methionine. Bacterial cultures supplemented with **9** (1050-fold lower k_{cat}/K_m) produce 57% as much mDHFR as cultures supplemented with methionine, and cultures supplemented with **2** (1850-fold lower k_{cat}/K_m) produce 28% of the control yield of protein. Bacterial cultures supplemented with **25** (4700-fold lower k_{cat}/K_m) do not support measurable levels of protein synthesis in this expression host; however, bacterial hosts exhibiting approximately 25-fold higher MetRS activity produce 23% as much mDHFR as cultures supplemented with methionine.[32] These results demonstrate that the rate of methionine analogue activation in vitro does indeed correlate with protein yield in vivo, and suggest that the kinetics of activation can play a critical role in controlling the rate of protein synthesis in methionine-depleted cultures supplemented with analogues that are poor substrates for MetRS.

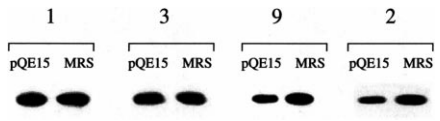


Figure 3. Western blot analysis of protein synthesis by bacterial expression hosts CAG18491/pQE15/pREP4 (pQE15) and CAG18491/pQE15-MRS/pREP4 (MRS). Bacterial cultures were supplemented with methionine, **2**, **3**, or **9**.

These results suggested that protein yields obtained from bacterial cultures supplemented with methionine analogues might be improved by increasing the MetRS activity of the bacterial host. To test this hypothesis, we compared the yields of protein prepared in the conventional bacterial expression host, CAG18491/pQE15/pREP4, to those obtained from a modified host, CAG18491/pQE15-MRS/pREP4. The modified host was prepared by transforming *E. coli* strain CAG18491 with the expression plasmid pQE15-MRS[32] and the repressor plasmid pREP4. The expression plasmid pQE15-MRS encodes MetRS under control of the *E. coli* promoter *metG p1* (Genbank accession number X55791)[50] as well as the target protein mDHFR. The MetRS activity of the bacterial hosts was determined as previously described,[32] with the modified host exhibiting 50-fold higher MetRS activity than the conventional strain (data not shown).

Protein synthesis was monitored for 5-ml cultures of these hosts supplemented with methionine or analogues **2**, **3**, or **9**; Western blot analyses of protein synthesis are shown in

Fig. 3. Although very low levels of protein synthesis were observed for negative control cultures of CAG18491/pQE15-MRS/pREP4, amino acid analyses, N-terminal sequencing, and NMR analyses of proteins produced in cultures of the modified host supplemented with **5** (the poorest of the substrates) still show 90–96% replacement of methionine by **5**.[32] Thus, the level of protein synthesis shown in Fig. 3 results from the incorporation of the analogue and is not due to incorporation of residual methionine. For cultures supplemented with methionine or **3**, the modified host, CAG18491/pQE15-MRS/pREP4, does not exhibit higher levels of protein synthesis than the conventional host CAG18491/pQE15/pREP4. Analysis by laser densitometry confirms these results, and reveals approximately equal accumulation of target protein for both strains; identical results have been obtained for large-scale expressions and purification of mDHFR. Activation of the analogue by MetRS does not appear to limit protein synthesis in cultures supplemented with **3**. For cultures supplemented with **2** or **9**, however, the modified bacterial host exhibits significantly increased levels of protein synthesis in comparison with the conventional host. Laser densitometry analysis indicates that the level of protein synthesis in the modified host is increased approximately 1.5-fold over that in the conventional host for cultures supplemented with **2**, and approximately 1.4-fold for cultures supplemented with **9**. Activation of these analogues by MetRS appears to limit protein synthesis in the conventional host, such that increasing the MetRS activity of the host is sufficient to restore high levels of protein synthesis. Indeed, preliminary results indicate that the yield of mDHFR obtained from large-scale cultures of CAG18491/pQE15-MRS/pREP4 supplemented with **2** is increased to approximately 35 mg/L (from 10 mg/L obtained from cultures of CAG18491/pQE15/pREP4 (Table 1)). Similar experiments are underway to determine the yields of protein obtained from cultures of CAG18491/pQE15-MRS/pREP4 supplemented with **9**. The results indicate that simple overexpression of MetRS can improve protein yields for cultures supplemented with methionine analogues that are poor substrates for MetRS, and may provide an attractive general method for efficient production of chemically novel protein materials in vivo.

Conclusion

Strategies to optimize the incorporation of non-natural amino acids into proteins in vivo are important for engineering protein materials. Based on our work to date, such strategies should include assessment of the AARS activities of the bacterial host; the rate of activation by MetRS plays a critical role in controlling the extent to which methionine analogues can be incorporated into proteins in vivo, and it seems likely that this situation will prove to be general. Quantitative assessment of the kinetics of activation by MetRS have indicated that even very poor substrates for the synthetase can be utilized by the protein synthesis machinery of a bacterial expression host. The correlation between the in vitro and in vivo results indicates the important role of the AARS and suggests that site-directed mutatgenesis and/or directed evolution of this class of enzymes may be used to increase further the number of non-natural amino acids that can be incorporated into proteins in vivo.

Our results also indicate that the kinetics of activation of methionine analogues by MetRS in vitro correlate with the level of protein synthesis supported by the analogues in vivo. The activity of the MetRS in the bacterial host can be manipulated, by overexpression of the MetRS, to improve the yields of proteins containing methionine analogues that are poor substrates for the MetRS. Overexpression of AARS may also be a general strategy for improving yields of proteins containing other non-natural amino acids, as well as proteins rich in particular natural amino acids. Manipulation of the AARS activities of a bacterial host has enormous potential for broadening the scope of protein engineering by permitting production of natural and artificial proteins with novel chemical and physical properties.

Experimental

Reagents

Each of the analogues **2**–**7** was prepared by alkylation of diethyl acetamidomalonate with the appropriate tosylate followed by decarboxylation and deprotection of the amine function.[26] Methionine and analogues **8** and **9** were obtained from Sigma. Radiolabeled sodium pyrophosphate was purchased from NEN Life Science Products, Inc., and isopropyl-β-D-thiogalactopyranoside was obtained from Calbiochem. The RGS-His antibody and anti-mouse IgG horseradish peroxidase conjugate used for Western blotting procedures were obtained from Qiagen and Amersham Life Sciences, respectively. All other reagents used during protein biosynthesis and purification and for activation assays were commercially available from Sigma, Aldrich, and Qiagen, and were used as received.

In vitro activation assays

The fully active, truncated form of MetRS was purified from overnight cultures of *E. coli* JM101 cells carrying the plasmid pGG3,[45] by using size exclusion methods previously reported.[51] Purified enzyme solutions (in 10 mM phosphate, pH 6.7, 10 mM β-mercaptoethanol) were concentrated to at least 3 μM prior to their storage in 40% glycerol at −20°C. Concentrations of enzyme stocks were determined by the Bradford method, using samples of MetRS quantified by amino acid analysis as standards. Activation of methionine analogs by MetRS was assayed via the amino acid-dependent ATP–PP_i exchange reaction at room temperature, also as previously described.[51,52] The assay, which measures the ^{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, and 2 mM PP_i (in the form of sodium pyrophosphate with a specific activity of approximately 0.5 TBq/mole)). Kinetic parameters for methionine analogues **2**, **3**, **5**, and **9** were obtained with an enzyme concentration of 75 nM and analogue concentrations of 100 μM to 20 mM. Parameters for methionine were obtained by using methionine

concentrations ranging from 10 μM to 1 mM. Aliquots (20 μl) were removed from the reaction mixture at various time points and 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 then resuspended in 0.5 mL of this solution and counted via liquid scintillation methods. Kinetic constants were calculated by a nonlinear regression fit of the data to the Michaelis–Menten model.

In vivo incorporation of non-natural amino acids

Buffers and media were prepared according to standard protocols.[53] 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 pQE15 and pREP4 (Qiagen), to obtain the expression host CAG18491/pQE15/pREP4. The auxotroph was transformed with the plasmids pQE15-MRS and pREP4 to obtain the modified bacterial expression host CAG18491/pQE15-MRS/pREP4. Both bacterial expression hosts produce the target protein mDHFR under control of a bacteriophage T5 promoter; the modified host also expresses extra copies of the MetRS gene under control of the constitutive *metG p1* promoter.[50]

Protein expression (1 liter scale). Similar procedures were used for preparation and isolation of mDHFR from media supplemented with the L-isomers of **1**, **2**, **3**, or **9**. 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 (35 mg/L) was inoculated with the appropriate *E. coli* strain (CAG18491/pQE15/pREP4 or CAG18491/pQE15-MRS/pREP4) and grown overnight at 37°C. This culture was used to inoculate 900 mL M9AA medium supplemented as described. The cells were grown to an optical density at 600 nm (OD_{600}) of approximately 0.9 and 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 600 mL of M9 medium. Cells were resuspended in 1000 mL of the M9AA medium described above, without methionine, and supplemented with 20 mg/L of the L-isomer of either **1**, **2**, **3**, or **9**. Protein synthesis was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Samples (1 mL) were collected after 4.5 h, the OD_{600} measured, and cells resuspended with distilled water to yield a normalized OD_{600} of 20. Protein expression was monitored by SDS polyacrylamide gel electrophoresis (12% acrylamide running gel); accumulation of mDHFR could be observed at an apparent molar mass of approximately 28 kDa after Coomassie staining.

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 (15,300 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.[49] 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) by batchwise dialysis against doubly distilled water for 4 days with at least 12 total changes of water. The dialysate was lyophilized to a purified powder of mDHFR. Experiments in M9AA medium afford approximately 30 mg of mDHFR for each of the bacterial expression hosts, while a control experiment in 2×YT medium afforded approximately 60 mg of mDHFR. Protein yields are reported as mg protein obtained per liter of bacterial culture; approximately 5–6 g of wet cells are obtained per liter of culture regardless of the identity of the analogue used to supplement the medium.

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 (35 mg/L) was inoculated with 5 mL of an overnight culture of the appropriate bacterial expression host. When the turbidity of the culture reached an 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 25 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 10 μL 10 mg/mL L-methionine (**1**) (positive control), L-homoallylglycine (**2**), L-homopropargylglycine (**3**), or L-norleucine (**9**), respectively. A culture lacking methionine (or any analogue) served as the negative control. Protein expression was induced by addition of IPTG to a final concentration of 0.4 mM. After 4 h, the OD_{600} was measured, and the samples were sedimented. After the supernatant was decanted, the cell pellets were resuspended in distilled water to yield a normalized OD of 20. Protein expression was monitored by SDS polyacrylamide gel electrophoresis (12% acrylamide running gel), followed by Western blotting. After transfer to a nitrocellulose membrane, Western blots were developed by treatment with a primary RGS-His antibody, followed by treatment with a secondary anti-mouse IgG conjugated to horseradish peroxidase to provide detection by chemiluminescence. Films were checked to ensure that band intensity was not saturated. Levels of protein synthesis were estimated by the intensity of the band on the gel, as determined using a Pharmacia Ultrascan XL laser densitometer and analysis by Pharmacia GelScan XL software. The accumulation of target protein is taken as evidence for incorporation of the non-natural amino acid, as **2**, **3**, **5**, and **9** have been shown to replace methionine, even in modified bacterial hosts, at levels of 92–98%.[25,26,30,32]

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