Identification of an expanded set of translationally active methionine analogues in *Escherichia coli*

K.L. Kiick[a], R. Weberskirch[b], D.A. Tirrell[b,*]

[a] *Department of Polymer Science and Engineering, University of Massachusetts, Amherst, MA 01003, USA*
[b] *Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA*

Received 6 June 2001; accepted 13 June 2001

First published online 9 July 2001

Edited by Lev Kisselev

Abstract Amino acid incorporation into proteins in vivo is controlled most stringently by the aminoacyl-tRNA synthetases. Here we report the incorporation of several new methionine analogues into protein by increasing the rate of their activation by the methionyl-tRNA synthetase (MetRS) of *Escherichia coli*. *cis*-Crotylglycine (4), 2-aminoheptanoic acid (7), norvaline (8), 2-butynylglycine (11), and allylglycine (12) will each support protein synthesis in methionine-depleted cultures of *E. coli* when MetRS is overexpressed and the medium is supplemented with the analogue at millimolar concentrations. These investigations suggest important opportunities for protein engineering, as expansion of the translational apparatus toward other amino acid analogues by similar strategies should also be possible. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Methionyl-tRNA synthetase; Protein engineering; Methionine; Non-natural amino acid

1. Introduction

The utilization of non-natural amino acids in the synthesis of novel proteins has attracted increasing scientific and technological interest. Strategies to incorporate non-natural amino acids into proteins in vivo have relied on the ability of the translational apparatus to accept a limited number of amino acid analogues with structures similar to those of the proteinogenic amino acids. Although an essentially unlimited number of non-natural amino acids can be incorporated into proteins via chemical methods such as solid-phase peptide synthesis and in vitro translation protocols [1,2], the incorporation of non-natural amino acids into proteins in vivo offers compelling advantages over these alternative methods. In addition to its synthetic simplicity and relatively high yields, the method affords multisite incorporation of non-natural amino acids, which has important consequences for protein behavior.

The fidelity of amino acid incorporation into proteins in vivo is controlled most stringently by the aminoacyl-tRNA synthetases (aaRS); the additional requirements of transport into the cell and recognition by the elongation factors and the ribosome [2,3] are generally more permissive than formation of aminoacyl-tRNA. For this reason, efforts to diversify the chemical composition of proteins by in vivo methods have increasingly focused on manipulation of the aaRS. Indeed, altering the aaRS activity of a bacterial host, by overexpression of wild-type [4], mutant [5], or heterologous synthetases [6,7], has expanded the repertoire of amino acids that can be used in protein biosynthesis.

Our continued interest in expanding the set of methionine analogues that can be utilized by the translational apparatus is motivated in part by the important role of methionine-rich interfaces in controlling protein–protein interactions [8,9]; replacement of methionine by chemically unique analogues may permit control or covalent trapping of these events. The study of methionine analogues may be uniquely fruitful in investigations of this kind, as the permissiveness of the *Escherichia coli* methionyl-tRNA synthetase (MetRS) has been demonstrated in previous reports of the translational activity of a variety of methionine analogues of varying chemical functionality and side chain length [10–13]. The critical role of MetRS in controlling the incorporation of methionine analogues into protein has been confirmed in studies in which the rates of activation of **2**–**9** in vitro have been correlated with the capacity of each of the analogues to support protein synthesis in vivo [4,14,15]. Here we present investigations of the activation and translational activity of methionine analogues **2**–**13** under a modified set of experimental conditions, and demonstrate that the translational apparatus can accept a larger set of methionine analogues than previously reported.

2. Materials and methods

2.1. Analogue synthesis

Each of the analogues **2**–**7** and **11** was prepared as previously described [14,16]. Analogues **8**, **9**, **12**, and **13** are available commercially (Sigma-Aldrich, St. Louis, MO, USA), and analogue **10** was a gift from Helen Blackwell (California Institute of Technology, Pasadena, CA, USA). Analogues **2**–**7** and **10**–**13** were used as the racemates in both in vivo and in vitro assays, and all concentrations given are for the L-isomers of the analogues.

2.2. Bacterial expression hosts

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/pQE15/pREP4. The plasmid pQE15 encodes the protein murine dihydrofolate reductase (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 analogues, and its expression is easily monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by Coomassie blue staining.

A modified bacterial expression host, CAG18491/pQE15-MRS/

*Corresponding author. Fax: (1)-626-793 8472.
E-mail address: tirrell@caltech.edu (D.A. Tirrell).

0014-5793/01/$20.00 © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(01)02657-6

pREP4, was prepared by transforming CAG18491 with plasmids pREP4 and pQE15-MRS. The plasmid pQE15-MRS encodes mDHFR, like pQE15, but also encodes the truncated form of MetRS [17] under control of the *E. coli* promoter *metGp1* (GenBank accession number X55791). Cultures of CAG18491/pQE15-MRS/pREP4 exhibit an approximately 50-fold increase in MetRS activity with respect to that of the conventional bacterial host CAG18491/pQE15/pREP4. These values are different from those reported previously for the bacterial hosts B834(DE3)/pQE15/pREP4 and B834(DE3)/pQE15-MRS/pREP4 [4], due to slight differences in the assay conditions used in determining the MetRS activity of the cell lysates.

The activity of the cell lysates was determined by adding 30 μl of cell lysate directly to solutions of the ATP–PP_i exchange reaction buffer, with a saturating concentration of methionine (750 μM), as previously described [4]. In this case, however, a control assay in which methionine was excluded was also analyzed for each cell lysate; the velocities obtained for the control assays were subtracted from the velocities observed for the cell lysates supplemented with methionine. This procedure corrected for ATP–PP_i exchange supported by the other 19 amino acid/aaRS pairs present in the cell lysate. A similar procedure conducted for the hosts B834(DE3)/pQE15/pREP4 and B834(DE3)/pQE15-MRS/pREP4 yielded a measured increase in MetRS activity of approximately 30-fold in the modified bacterial host with respect to the conventional host.

2.3. Determination of translational activity

Buffers and media were prepared according to standard protocols. 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 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 to remove methionine from the cell culture. The cells were sedimented, the supernatant was removed, 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 60 mg/l or 500 mg/l of methionine or **2**–**13**. A culture lacking methionine (or any analogue) 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, and cultures were grown for 4.5 h. Protein expression was monitored by SDS–PAGE of samples with normalized OD_{600} and visualized by Coomassie blue staining. The accumulation of the target protein mDHFR was taken as evidence for incorporation of the non-natural amino acid. Incorporation was confirmed by analyzing purified protein via amino acid analysis or N-terminal sequencing.

2.4. Large scale protein expression

Larger scale production of mDHFR was conducted in 1-l or 50-ml cultures of CAG18491/pQE15-MRS/pREP4, as described above. After the medium shift, however, the cultures were resuspended in either 1 l of M9AA medium lacking methionine and containing 500 mg/l of **11**, or in 50 ml of M9AA medium lacking methionine and supplemented with 500 mg/l of **4**, **7**, **8**, or **12**. Protein expression was induced as described above. These larger scale cultures were sedimented after 4.5 h, and the cell pellet was stored at −80°C overnight.

2.5. Protein purification

mDHFR was purified from the cell pellet via 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 500 mg/l of **11**, similar to the yield obtained for mDHFR from cultures supplemented with methionine. Similar purification procedures were used for the cell pellets of the 50-ml cultures supplemented with 500 mg/l of **4**, **7**, **8**, or **12**. Protein yields obtained from these cultures ranged from 50 to 250 μg/50 ml.

2.6. Activation of methionine analogues in vitro

The fully active, truncated form of the wild-type MetRS was purified from 24-h cultures of JM101 cells carrying the plasmid pGG3 [17]. (The pGG3 plasmid was kindly donated by Professor Hieronim Jakubowski of UMDNJ-New Jersey Medical School, Newark, NJ, USA.) The plasmid pGG3 encodes MetRS under control of the *E. coli* promoter *metGp1*. The enzyme was purified by size exclusion chromatography as previously described [18].

Activation of methionine analogues by wild-type MetRS was assayed via the amino acid-dependent ATP–PP_i exchange reaction, also as previously described [15,18,19]. 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 bovine serum albumin, and 2 mM PP_i (in the form of sodium pyrophosphate (NEN Life Science Products, Inc.) with a specific activity of 0.2–0.5 TBq/mol)). Assays to determine if the methionine analogues **2**–**13** support PP_i exchange catalyzed by MetRS were conducted in solutions 75 nM in enzyme and 5 mM in the L-isomer of the analogue with a reaction time of 20 min.

Kinetic parameters for homoallylglycine (**2**), homopropargylglycine (**3**), *trans*-crotylglycine (**5**), and norleucine (**9**) have previously been reported [15]. Quantitative kinetic parameters for **11** were obtained with an enzyme concentration of 75 nM and analogue concentrations of 100 μM to 20 mM. For analogue **8**, an enzyme concentration of 60 nM and analogue concentrations of 2.5–35 mM were used in the ATP–PP_i exchange assay. Analogues **4** and **12** are such poor substrates that only k_{cat}/K_m values could be determined accurately. For **12**, enzyme concentrations of 100–150 nM and analogue concentrations of 5–50 mM were used. The lower solubility of **4** precluded the use of these high analogue concentrations, so the k_{cat}/K_m value was estimated by calculating the slope of the plot of velocity vs. substrate concentration at low substrate concentration [20,21]. Enzyme concentrations of 150 nM and analogue concentrations of 0.6–6.6 mM were used. Measurable velocities could not be obtained for analogue **7**, owing to the very low solubility of this analogue at the pH of the enzyme assay reaction buffer. Parameters were not determined for 6,6,6-trifluoro-2-aminohexanoic acid (**6**), *O*-allylserine (**10**), or propargylglycine (**13**), as these analogues did not support measurable PP_i exchange under any assay conditions. Parameters for methionine were obtained by using concentrations ranging from 10 μM to 1 mM. Kinetic constants were calculated by non-linear regression fit of the data to a Michaelis–Menten model.

3. Results

3.1. Activation and translational activity of methionine analogues

Previous investigations of the translational activity of methionine analogues **2**–**9** indicated that **2**, **3**, and **9** support

Table 1
Kinetic parameters for methionine analogues in the ATP–PP_i exchange reaction

Analogue	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	k_{cat}/K_m (Rel)[a]
1	24.3 ± 2	13.3 ± 0.2	5.5×10^{-1}	1
3	2 415 ± 170	2.60 ± 0.3	1.1×10^{-3}	1/500
9	4 120 ± 900	2.15 ± 0.6	5.2×10^{-4}	1/1 050
2	4 555 ± 200	1.35 ± 0.1	3.0×10^{-4}	1/1 850
5	15 675 ± 250	1.82 ± 0.6	1.2×10^{-4}	1/4 700
11	38 650 ± 2 000	1.51 ± 0.5	3.9×10^{-5}	1/14 000

[a]Relative to methionine.

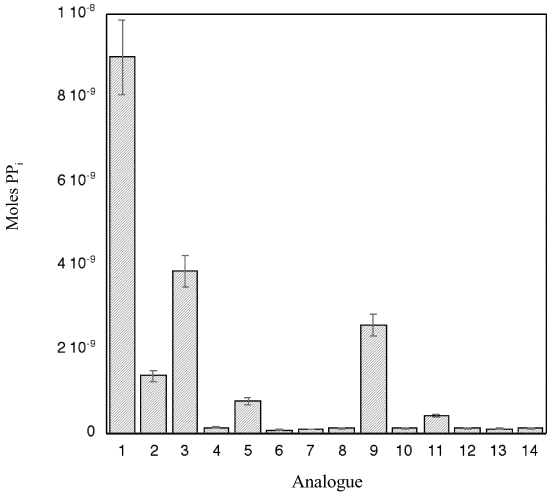

Fig. 1. Activation of methionine and methionine analogues by MetRS. The amount of PP_i exchanged in 20 min, as measured in the ATP–PP_i exchange assay, is shown for 5 mM solutions of methionine (**1**) and analogues **2**–**13**. The background (**14**) is given for a reaction mixture lacking enzyme and amino acid.

protein synthesis in the conventional bacterial expression host CAG18491/pQE15/pREP4 [14]. Accordingly, **2**, **3**, and **9** support the highest rates of in vitro PP_i exchange. Analogue **5** supports measurable, but lower levels of exchange, and overexpression of MetRS is required to rescue its translational activity [4]. In order to assess the ability of analogues **10**–**13** to serve as methionine surrogates in vivo, activation of the analogues by MetRS in vitro was monitored via the ATP–PP_i exchange assay. Results for these studies are shown in Fig. 1, with results for analogues **2**–**9** given for comparison. Of the analogues **10**–**13**, only **11** is indicated to support PP_i exchange under the assays conditions, at a level even lower than that observed for **5**.

The kinetic parameters for **11** were therefore determined, yielding the values shown in Table 1. Previously determined values for methionine and analogues **2**, **3**, **5**, and **9** [15] are shown for comparison. Our measured value of K_m for methionine matched previously reported values [22], although the value determined for k_{cat} was slightly lower than that reported. Comparison of the k_{cat}/K_m value for **11** (3.9×10^{-5} s^{-1} μM^{-1}) with values for methionine and the other analogues demonstrates that **11** is indeed a much poorer substrate for MetRS, with a catalytic efficiency in activation 14 000-fold poorer than that for methionine. As the translational activity of **5**, which has a catalytic efficiency 4700-fold lower than that for methionine, could be rescued by overexpression of MetRS in a bacterial host, it seemed likely that a similar result should be observed for **11**.

In order to test this hypothesis, bacterial hosts suitable for testing the translational activity of methionine analogues **2**–**8** and **10**–**13** were prepared by transformation of *E. coli* strain CAG18491, a methionine auxotroph, with the repressor plasmid pREP4 and with either of the two expression plasmids pQE15 or pQE15-MRS (Section 2). The translational activity of each analogue was assessed on the basis of its capacity to support synthesis of mDHFR in methionine-depleted cultures of CAG18491/pQE15/pREP4 or CAG18491/pQE15-MRS/pREP4; the results are shown in Fig. 2.

The target protein was not observed in the negative control culture of CAG18491/pQE15/pREP4, or in cultures of CAG18491/pQE15/pREP4 or CAG18491/pQE15-MRS/pREP4 supplemented with **4**, **6**, **7**, **8**, **10**, **12**, or **13**. In contrast, mDHFR was detected in both bacterial host cultures supplemented with methionine (**1**), **2**, **3**, or **9**, as indicated by the appearance of a protein band at the position expected for mDHFR in SDS–PAGE, and consistent with our previous investigations. For the negative control cultures and for cultures supplemented with **5** and **11**, however, the behavior of the bacterial hosts differed, as shown in Fig. 2. The target protein mDHFR was not detected in the CAG18491/pQE15/pREP4 cultures supplemented with **5** or **11** (Fig. 2A), while strong induction of mDHFR was observed for CAG18491/pQE15-MRS/pREP4 under the same conditions (Fig. 2B). Even the unsupplemented control culture of CAG18491/pQE15-MRS/pREP4 shows evidence of mDHFR synthesis, suggesting that introduction of pQE15-MRS does indeed increase the rate of activation of methionine in the modified host. While we have previously reported the translational activity of **5** under these experimental conditions, these results indicate that overexpression of MetRS can rescue the translational activity of **11** as well, despite the fact that it is a 14 000-fold poorer substrate for MetRS than methionine.

Supplementation by **11** at a concentration of 60 mg/l was required to observe measurable accumulation of target protein, while supplementation with 20 mg/l was sufficient to observe the same results for **5**. These observations are consistent with the fact that **11** is a poorer substrate for MetRS than **5** (Table 1) and with the supposition that formation of the aminoacyl-tRNA is consequently the rate-limiting step in protein synthesis in the *E. coli* host. Furthermore, raising the concentration of **11** in the culture medium to 500 mg/l (3.9 mM) improves protein yields (data not shown), suggesting that the rate of activation of **11** is increased upon raising its concentration in the medium, presumably by raising the intra-

Table 2
Extent of replacement of methionine by methionine analogues

Analogue	Extent of replacement (%)	
	Amino acid analysis	N-terminal sequencing
11	98 ± 2	94 ± 3
8	92 ± 3	87 ± 2
12	84 ± 5	84 ± 2
4	76 ± 5	79 ± 5
7	60 ± 5	56 ± 5

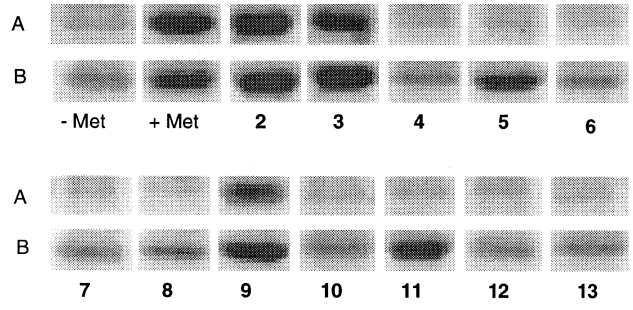

Fig. 2. SDS–PAGE analysis of mDHFR expression in bacterial cultures supplemented with methionine analogues at 60 mg/l, as indicated. A: The conventional bacterial host CAG18491/pQE15/pREP4. B: The modified bacterial host CAG18491/pQE15-MRS/pREP4.

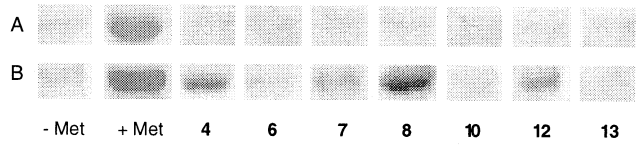

Fig. 3. SDS–PAGE analysis of mDHFR synthesis in bacterial cultures supplemented with methionine analogues at 500 mg/l. Cultures were supplemented with methionine, **4**, **6**, **7**, **8**, **10**, **12**, and **13**, as indicated. A: The conventional host CAG18491/pQE15/pREP4. B: The modified host CAG18491/pQE15-MRS/pREP4.

cellular concentration of the analogue. We were unable to measure the increase in intracellular concentration of the analogue by amino acid analysis, as the analogue is not stable under the analysis conditions. Still, it seemed likely that the intracellular concentration, and therefore rate of activation, of the other methionine analogues could be raised similarly. The translational activity of each of the analogues **4**, **6**–**8**, **10**, **12**, and **13** was therefore tested in bacterial cultures supplemented with 500 mg/l of each compound.

3.2. Incorporation of additional methionine analogues

Expression of the target protein mDHFR was monitored by SDS–PAGE analysis for both conventional (CAG18491/pQE15/pREP4) and modified (CAG18491/pQE15-MRS/pREP4) bacterial expression hosts with supplementation at 500 mg/l of **4**, **6**, **7**, **8**, **10**, **12**, and **13** (3.8–4.3 mM). The results from SDS–PAGE analysis of these cultures are shown in Fig. 3. Negative control cultures did not show any target protein synthesis, while both positive control cultures supported protein synthesis in vivo. No protein synthesis was observed in cultures of the conventional bacterial host supplemented with the analogues at high concentration (Fig. 3A). For the modified bacterial host (Fig. 3B), however, the accumulation of target protein was observed in cultures supplemented with 500 mg/l of analogues **4**, **7**, **8**, or **12**. Increasing the concentration of amino acid in the medium, coupled with overexpression of MetRS, is required for these methionine analogues to support protein biosynthesis.

3.3. Protein characterization

Amino acid analysis of purified proteins produced from cultures supplemented with 500 mg/l of **4**, **7**, **8**, **11**, and **12** was conducted to confirm replacement of methionine by these analogues. Amino acid analysis shows a decrease in methionine content from the expected value of 3.8 mol% in proteins produced from cultures supplemented with the analogues. It is impossible to detect the analogues directly by amino acid analysis, owing to their instability under the analysis conditions. If, however, depletion of methionine is assumed to result from replacement by the analogue, the observed analyses indicate overall extents of incorporation for the analogues ranging from 60% to 98%, as shown in Table 2. Each of the amino acids is within experimental error (±10%) of the theoretical values for mDHFR, with the exception of the decrement in methionine content. This suggests that none of the other 19 amino acids competes effectively with the analogues in replacing methionine.

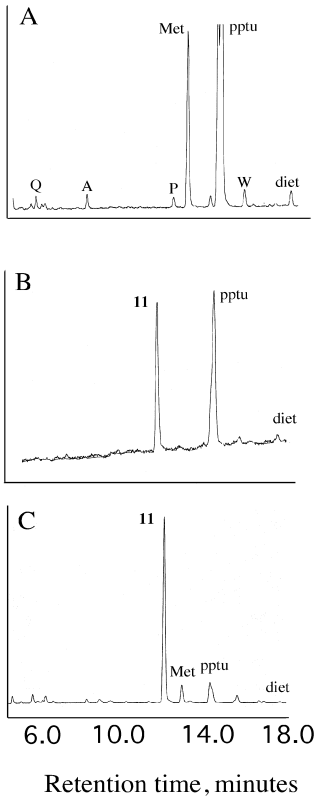

Fig. 4. N-terminal sequencing results indicating occupancy of the initiator site in DHFR produced in bacterial cultures supplemented with **11**. N-terminal residues are shown for A: DHFR-Met, B: the free amino acid **11**, and C: DHFR-**11**, as determined by Edman degradation.

Retention of the N-terminal (initiator) methionine in mDHFR is expected on the basis of the identity of the penultimate amino acid [23], so N-terminal sequencing provided an additional means of assessing the extent of replacement of methionine by the analogues. Because the analogues are not degraded under the analysis conditions, they can be detected directly, and this analysis provides direct evidence for the presence of the analogues in the target protein. Results for mDHFR produced from cultures supplemented with 500 mg/l of **11** (mDHFR-**11**) are shown in Fig. 4. The signal corresponding to methionine elutes at 13.0 min, while that for **11** elutes at 11.7 min. The large peaks that elute at approximately 14.7 min correspond to piperidylphenylthiourea, a product of the analysis resulting from the buffer, and the small peak at

Table 3
Kinetic parameters for activation of methionine analogues **4**, **8**, and **12** by MetRS

Analogue	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	k_{cat}/K_m (Rel)[a]
8	14 380 ± 900	0.17 ± 0.05	1.2×10^{-5}	1/45 600
4	ND[b]	ND	4.3×10^{-6}	1/127 000
12	ND	ND	1.6×10^{-6}	1/340 000

[a]Relative to methionine.
[b]Not determined.

approximately 18.5 min corresponds to diethylphthalate, an internal standard. Comparison of chromatograms of the N-terminal residues of mDHFR and mDHFR-**11** demonstrates that the methionine that normally occupies the initiator position of mDHFR (Fig. 4A) is replaced with **11** (Fig. 4B) in mDFHR-**11** (Fig. 4C). Similar results were observed for the analogues **4**, **7**, **8**, and **12**. These results clearly indicate the incorporation of a variety of methionine analogues at the initiator site of mDHFR. Integration of the peak areas corresponding to methionine and to the analogues indicates up to 94% replacement of methionine; results are given for each analogue in Table 2.

3.4. Activation of methionine analogues by MetRS in vitro

Activation of methionine analogues **4**, **7**, **8**, and **12** by MetRS was assayed by the ATP–PP_i exchange assay as previously described. Previous characterization of the exchange supported by **4**, **7**, **8**, or **12** (Fig. 1) indicated that none of them supported measurable PP_i exchange at 20 min at an analogue concentration of 5 mM. In these experiments, analogues at concentrations of 15 mM were incubated with the enzyme for 3.5 h prior to quenching. The activation of **7** could not be measured under these conditions owing to its insolubility at high concentrations. Analogues **4**, **8**, and **12** support PP_i exchange at levels greater than background under these experimental conditions, while analogues **6**, **10** and **13** do not (data not shown). The kinetic parameters for **4**, **8**, and **12** were therefore measured as previously described. As shown in Table 3, the analogues are increasingly poor substrates for the enzyme, with k_{cat}/K_m values up to 340 000-fold lower than those observed for methionine.

4. Discussion

Combined with our previous results detailing the incorporation of **2**, **3**, and **5**, the incorporation of **4**, **7**, **8**, **11**, and **12** into proteins in vivo demonstrates the promiscuity of the translational apparatus toward methionine analogues under appropriate experimental conditions. Increasing the MetRS activity of the cellular host and/or increasing the concentration of analogue in the medium increases the rate of activation of analogues **4**, **7**, **8**, **11**, and **12** sufficiently to rescue their translational activity in vivo. These procedures permit the incorporation of methionine analogues that are up to 340 000-fold poorer substrates for MetRS in activation than methionine, demonstrating that much poorer substrates than previously imagined can be utilized by the protein biosynthesis machinery under appropriate experimental conditions.

Understanding the features of the analogue that are important for activation and incorporation may facilitate not only the rational design of additional novel methionine analogues, but also the design of mutant forms of MetRS. In these and previous studies, the importance of side chain length in facilitating activation of an analogue by MetRS is illustrated, as **7**, **8**, **10**, **12**, and **13** are activated much less efficiently by MetRS than **2**, **3**, **5**, **9**, and **11** (Table 1). Although **4** has the same side chain length as methionine, the conformational restriction placed on the side chain by the *cis*-conformation of the double bond imposes on **4** a geometry very different from that of methionine bound at the active site of MetRS [24]. The importance of p- or π-electron density in the amino acid side chain is suggested by the fact that the methionine thioether makes two hydrogen bonds – one with the side chain of Tyr260 and another with the backbone NH of Leu13 – in the active site of MetRS [24]. Therefore, although the geometries or side chain lengths of **4**, **11**, and **12** are different from those of methionine, the availability of π-electrons near the δ-position of these analogues is likely to play a role in their activation by MetRS. The presence of π-electrons near the δ-positions of **3** and **11** is almost certainly critical for activation, given the side chain geometries of these analogues [14], and we have recently found that the availability of electron density at the δ-position is essential in the activation of azido amino acids [25].

The observed promiscuity of MetRS may result not only from structural and electronic similarities of the methionine analogues examined here, but also from certain unique features of the enzyme. Comparison of the crystal structures of the free [26] and methionine-bound [24] forms of MetRS shows that the active site undergoes significant conformational changes upon methionine binding; this flexibility may be important in enabling MetRS to activate methionine analogues with varying side chain lengths and chemical functionality. Furthermore, MetRS does not exhibit general editing mechanisms common to other aaRS, which may permit MetRS to charge $tRNA^{Met}$ with a broad range of non-natural amino acids. Indeed, mutation of residues in the editing site of valyl-tRNA synthetase permits incorporation of aminobutyrate in place of valine in vivo [27], and similar strategies to eliminate editing activity in other aaRS appear likely to prove useful in expanding the synthetic versatility of the translational apparatus.

The conformational flexibility of the active site and the lack of general editing by MetRS suggest that this enzyme might be particularly suited for directed evolution strategies aimed at incorporating novel methionine analogues into proteins in vivo. Because even extremely poor substrates for MetRS can support protein synthesis under appropriate conditions, even modest gains in the rates of activation of analogues by MetRS may be sufficient to permit incorporation of new methionine analogues by the methods described here. Similar strategies will likely be generally applicable to all aaRS. Methods to expand the set of translationally active methionine analogues via directed evolution are currently under investigation.

The results reported here illustrate one of an increasing number of strategies for the incorporation of non-natural amino acids into proteins in vivo. Given the many methods by which one can tailor the aaRS activity of a bacterial host, the prospects appear promising for substantial broadening of the scope of protein engineering in vivo through the incorporation of chemically novel amino acids.

Acknowledgements: This work was supported by grants from the Polymers and Genetics Programs of the U.S. National Science Foundation and from the U.S. Army Research Office. We are grateful to J.C.M. van Hest and to H. Blackwell for synthesis of methionine analogues and to H. Jakubowski and Y. Mechulam for donation of plasmids encoding MetRS. K.L.K. thanks the U.S. Department of Defense for a National Defense Science and Engineering Graduate Fellowship.

References

[1] Hecht, S.M., Alford, B.L., Kuroda, Y. and Kitano, S. (1978) J. Biol. Chem. 253, 4517–4520.

[2] Cornish, V.W., Mendel, D. and Schultz, P. (1995) Angew. Chem. Int. Ed. Engl. 34, 621–633.
[3] Mendel, D., Ellman, J.A., Chang, Z., Veenstra, D.L., Kollman, P.A. and Schultz, P.G. (1992) Science 256, 1798–1802.
[4] Kiick, K.L., van Hest, J.C.M. and Tirrell, D.A. (2000) Angew. Chem. Int. Ed. Engl. 39, 2148–2152.
[5] Sharma, N., Furter, R., Kast, P. and Tirrell, D.A. (2000) FEBS Lett. 467, 37–40.
[6] Furter, R. (1998) Protein Sci. 7, 419–426.
[7] Wang, L., Brock, A., Herberich, B. and Schultz, P.G. (2001) Science 292, 498–500.
[8] Yuan, T. and Vogel, H.J. (1999) Protein Sci. 8, 113–121.
[9] Schenck, H.L., Dado, G.P. and Gellman, S.H. (1996) J. Am. Chem. Soc. 118, 12487–12494.
[10] Cowie, D.B. and Cohen, G.N. (1957) Biochim. Biophys. Acta 26, 252–261.
[11] Budisa, N., Steipe, B., Demange, P., Eckerskorn, C., Kellermann, J. and Huber, R. (1995) Eur. J. Biochem. 230, 788–796.
[12] Duewel, H., Daub, E., Robinson, V. and Honek, J.F. (1997) Biochemistry 36, 3404–3416.
[13] Jakubowski, H. (2000) J. Biol. Chem. 275, 21813–21816.
[14] van Hest, J.C.M., Kiick, K.L. and Tirrell, D.A. (2000) J. Am. Chem. Soc. 122, 1282–1288.
[15] Kiick, K.L. and Tirrell, D.A. (2000) Tetrahedron 56, 9487–9493.
[16] van Hest, J.C.M. and Tirrell, D.A. (1998) FEBS Lett. 428, 68–70.
[17] Ghosh, G., Brunie, S. and Schulman, L.H. (1991) J. Biol. Chem. 266, 17136–17141.
[18] Mellot, P., Mechulam, Y., LeCorre, D., Blanquet, S. and Fayat, G. (1989) J. Mol. Biol. 208, 429–443.
[19] Ghosh, G., Pelka, H. and Schulman, L.D. (1990) Biochemistry 29, 2220–2225.
[20] Fersht, A. (1999) Enzyme Structure and Mechanism, W.H. Freeman and Company, New York.
[21] Kim, H.Y., Pelka, H., Brunie, S. and Schulman, L.H. (1993) Biochemistry 32, 10506–10511.
[22] Kim, H.Y., Ghosh, G., Schulman, L.H., Brunie, S. and Jakubowski, H. (1993) Proc. Natl. Acad. Sci. USA 90, 11553–11557.
[23] Hirel, P.H., Schmitter, J.M., Dessen, P., Fayat, G. and Blanquet, S. (1989) Proc. Natl. Acad. Sci. USA 86, 8247–8251.
[24] Serre, L., Verdon, G., Choinowski, T., Hervouet, N., Risler, J.-L. and Zelwer, C. (2001) J. Mol. Biol. 306, 863–876.
[25] Kiick, K.L., Saxon, E., Tirrell, D.A. and Bertozzi, C.B. (2001), in preparation.
[26] Mechulam, Y., Schmitt, E., Maveyraud, L., Zelwer, C., Nureki, O., Yokoyama, S., Konno, M. and Blanquet, S. (1999) J. Mol. Biol. 294, 1287–1297.
[27] Döring, V., Mootz, H.D., Nangle, L.A., Hendrickson, T.L., de Crécy-Lagard, V., Schimmel, P. and Marlière, P. (2001) Science 292, 501–504.