A COMPARISON OF ETHIONINE WITH METHIONINE IN *ESCHERICHIA COLI* IN VITRO POLYPEPTIDE CHAIN INITIATION AND SYNTHESIS

J. M. OLD* and D. S. JONES

Department of Biochemistry, University of Liverpool, P.O. Box 147 Liverpool L69 3BX, UK

Received 19 May 1976

1. Introduction

Several analogues of methionine can successfully replace methionine in the reactions of protein synthesis in *Escherichia coli*. For example ethionine, norleucine and selenomethionine have been shown to support ATP–PP_i exchange [1] and to be esterified to tRNA [2]. Trupin et al. [2] also showed that norleucyl-tRNA and ethionyl-tRNA could be formylated and Kerwar and Weissbach [3] showed that formylnorleucyl-tRNA behaved similarly to formylmethionyl-tRNA in the initiation of protein synthesis. However, the experiments of Trupin et al. and Kerwar and Weissbach were performed with unfractionated tRNA, with which a proper study of the kinetic parameters of the formylation reaction cannot be made.

Using highly purified species of *E. coli* $tRNA_f^{Met}$ and $tRNA_m^{Met}$ we have compared the recognition of ethionine with methionine in the aminoacylation reaction and the formylation reaction by determining the kinetic parameters for these reactions. We have also studied the ability of formylethionyl-$tRNA_f^{Met}$ to initiate polypeptide chain synthesis in vitro as directed by poly AUG. We report here that ethionine can be charged to both $tRNA_f^{Met}$ and $tRNA_m^{Met}$ but is recognised to a lesser extent than methionine by methionyl-tRNA synthetase, that ethionyl-$tRNA_f^{Met}$ is recognised and formylated to the same extent as methionyl-$tRNA_f^{Met}$ by the transformylase enzyme and that formylethionyl-$tRNA_f^{Met}$ can initiate both polymethionine and polyethionine synthesis to the same extent as formylmethionyl-$tRNA_f^{Met}$.

*Present address: Nuffield Department of Clinical Medicine, The Radcliffe Infirmary, Oxford, UK.

Abbreviations: Eth, ethionyl; f-Met, formyl-methionyl; f-Eth, formyl-ethionyl. Poly d-ATC:GAT refers to the DNA-like deoxyribonucleotide polymer which contains the repeating trinucleotide deoxyadenylyl–thymidyl–cytidylate in one strand and repeating trinucleotide deoxyguanylyl–deoxyadenylyl–thymidylate in the complementary strand. Poly AUG refers to a ribopolynucleotide containing the repeating trinucleotide sequence, adenylyl–uridylyl–guanylate.

2. Materials and methods

L-[^{14}C]Ethionine (54 mCi/mmole), L-[^{14}C] methionine (54 mCi/mole) and sodium [^{3}H]formate (250 mCi/mmole) were purchased from the Radiochemical Centre, Amersham. Pure *E. coli* methionyl-tRNA was a gift from Dr C. J. Bruton (Cambridge). Unfractionated *E. coli* tRNA was a gift from Dr S. Nishimura (Tokyo). Purified species of *E. coli* $tRNA_f^{Met}$ and $tRNA_m^{Met}$ were prepared by DEAE-Sephadex chromatography followed by arginine-Sepharose chromatography of tRNA [4]. Aminoacyl-tRNAs and ^{14}C-labelled aminoacyl-tRNAs were prepared as described by Nishimura et al. [5].

Unlabelled and ^{3}H-labelled $N^{5,10}$-methenyltetrahydrofolic acid were synthesised from either sodium formate or sodium [^{3}H]formate and tetrahydrofolic acid using formyltetrahydrofolate synthetase [6] which was contained in an extract from *Clostridium acidi-urici*, a gift from Dr J. C. Rabinowitz (University of California). The methenyltetrahydrofolate products were purified by cellulose chromatography according to Huennekens [7]. N^{10}-Formyltetrahydrofolic acid was obtained by neutralisation of the $N^{5,10}$-methenyl derivative with dilute ammonia immediately before

North-Holland Publishing Company – Amsterdam

use. A crude extract from *E. coli* [5] was used as a transformylase preparation.

Polymethionine and polyethionine synthesis was studied using a modification of the two step reaction procedure described by Ghosh et al. [8]. In the first step, poly AUG was synthesised by RNA polymerase (a gift from Dr V. Paetkau, University of Alberta) using the DNA template poly d-ATC:GAT (a gift from Professor N. K. Gupta, University of Nebraska). In step two, the poly AUG was used without isolation for polypeptide synthesis using washed ribosomes and a 100 000 g supernatant fraction from *E. coli* strain MRE 600 [5].

3. Results

Table 1 shows the kinetic parameters of the aminoacylation of $tRNA_f^{Met}$ and $tRNA_m^{Met}$ with methionine and ethionine by pure methionyl-tRNA synthetase. The K_M and K_i values were calculated from the reciprocal plots of the initial velocities and the substrate concentrations according to Lineweaver and Burk [10]. The K_M values for methionine with $tRNA_f^{Met}$ was found to be very similar to the value with $tRNA_m^{Met}$ whereas the K_m values for ethionine with each tRNA species were different and were also an order of magnitude higher than those for methionine. Ethionine was found to be a competitive inhibitor of the charging of methionine, exhibiting a similar K_i value with either tRNA species.

Fig.1 shows a reciprocal plot of the formylation of methionyl-$tRNA_f^{Met}$ and also of ethionyl-$tRNA_f^{Met}$. The results in fig.1 show that both substrates exhibited identical reaction kinetics in the formylation reaction. The K_M for both substrates was found to be 0.3 μM which agrees with the value reported for methionyl-$tRNA_f^{Met}$ by Giegé et al. [11] of 0.6 μM.

The results of the experiments to study the ability of formylethionyl-$tRNA_f^{Met}$ to initiate polypeptide synthesis are shown in table 2. The addition of formylethionyl-$tRNA_f^{Met}$ was found to increase polymethionine synthesis from 57 to 113 pmol/ml, a level slightly higher than that obtained by the addition of formylmethionyl-$tRNA_f^{Met}$. A similar result was obtained for the initiation of polyethionine synthesis although the amount of polyethionine synthesised was less than that of polymethionine. The results in table 2 show that formylethionyl-$tRNA_f^{Met}$ addition caused an increase from 39 to 87 pmol of polyethionine synthesised per ml, compared to 65 pmol/ml with formylmethionyl-$tRNA_f^{Met}$. These results suggested that ethionine-initiated polypeptide synthesis more efficiently than methionine. This was confirmed

Table 1
The kinetic parameters of the aminoacylation of $tRNA_f^{Met}$ and $tRNA_m^{Met}$

Substrate	tRNA	K_M (μM)	V_{max}	K_i (mM)
[^{14}C]Met	$tRNA_f^{Met}$	1.2	25	–
[^{14}C]Met	$tRNA_m^{Met}$	1.4	50	–
[^{14}C]Eth	$tRNA_f^{Met}$	13.0	8	–
[^{14}C]Eth	$tRNA_m^{Met}$	70.0	45	–
[^{14}C]Met + Eth	$tRNA_f^{Met}$	–	25	0.15
[^{14}C]Met + Eth	$tRNA_m^{Met}$	–	50	0.19

The incubation mixture consisted of 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.05 mM EDTA, 25 mM ATP and contained 0.25 A_{260} units of $tRNA_f^{Met}$ or $tRNA_m^{Met}$, [^{14}C]methionine (10^{-5} to 5×10^{-7} M) or [^{14}C]ethionine (10^{-4} to 5×10^{-6} M), 1.5 μg of pure methionyl-tRNA synthetase and, where indicated, cold ethionine (2×10^{-4} M) in a total volume of 250 μl. After 2 min incubation at 37°C a 200 μl sample was removed and assayed for [^{14}C]aminoacyl-tRNA formation as described by Old and Jones [9]. Maximum velocities are expressed as nmoles of methionine or ethionine incorporated into tRNA per min per mg of protein.

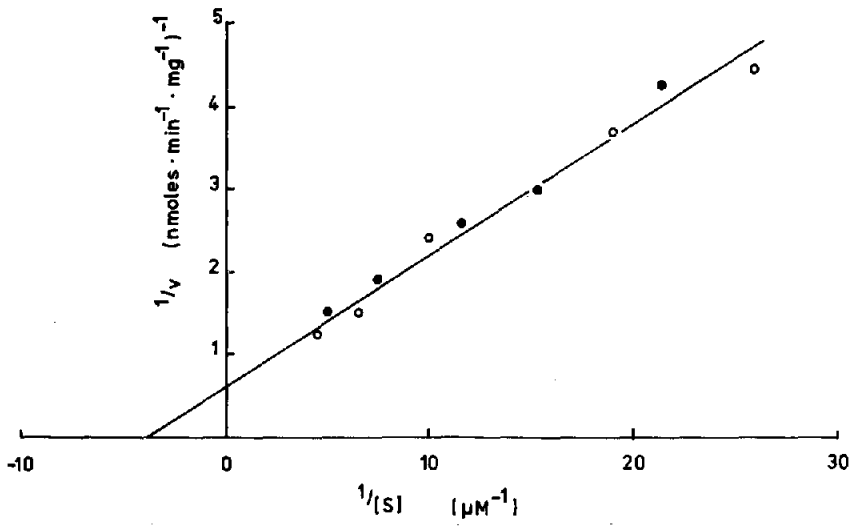

Fig.1. Reciprocal plots for the formylation of Met-tRNA$_f^{Met}$ (•) and Eth-tRNA$_f^{Met}$ (○) The reaction mixtures consisted of 25 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 0.5 mM EDTA, 25 mM ATP and contained different amounts of either Met-tRNA$_f^{Met}$ or Eth-tRNA$_f^{Met}$, 1 μmol of freshly neutralised [^{3}H]$N^{5,10}$-methenyltetrahydrofolic acid and 0.19 mg of crude extract from *E. coli* in a total volume of 250 μl. After 3 min incubation at 37°C, a 200 μl sample was removed and assayed for formylaminoacyl-tRNA by the filter paper disc method according to Old and Jones [9].

Table 2
Incorporation of methionine and ethionine from [^{14}C]aminoacyl-tRNA$_m^{Met}$ into polymethionine and polyethionine

Step 2 components	Polypeptide synthesis (pmoles/ml)
[^{14}C]Met-tRNA$_m^{Met}$	57
[^{14}C]Met-tRNA$_m^{Met}$ + f Eth-tRNA$_f^{Met}$	113
[^{14}C]Met-tRNA$_m^{Met}$ + f Met-tRNA$_f^{Met}$	89
[^{14}C]Eth-tRNA$_m^{Met}$	39
[^{14}C]Eth-tRNA$_m^{Met}$ + f Eth-tRNA$_f^{Met}$	87
[^{14}C]Eth-tRNA$_m^{Met}$ + f Met-tRNA$_f^{Met}$	65

The step one reaction mixture contained 5 μmol of Tris-HCl pH 7.8, 0.5 μmol of $MgCl_2$, 0.125 μmol of $MnCl_2$, 1.5 μmol of mercaptoethanol, 0.04 μmol of UTP, GTP and ATP respectively, 0.02 A_{260} units of poly d-ATC:GAT and 10 μg of RNA polymerase in a total volume of 125 μl. After 2 h incubation at 37°C, the reaction mixture was chilled and supplemented with the components of a protein synthesis system. The step two reaction mixture contained 15 μmol Tris-HCl, pH 7.8, 0.5 μmol of $MgCl_2$, 2.3 μmol of magnesium acetate, 4 μmol of KCl, 0.5 μmol of ATP, 0.06 μmol of GTP, 1.25 μmol of phosphoenolpyruvate, 1 μg of pyruvate kinase, 4 A_{260} units of *E. coli* ribosomes, 2.0 A_{260} units of [^{14}C]aminoacyl-tRNA, 1.0 A_{260} unit for formylaminoacyl-tRNA as indicated and the poly d-ATC:GAT, excess nucleoside triphosphates and RNA polymerase from step one, in a total volume of 250 μl. After 10 min incubation at 0°C, 0.2 mg of *E. coli* 100 000 *g* supernatant was added and the mixture incubated at 37°C. After 15 min incubation a 200 μl was removed and assayed for [^{14}C] polypeptide synthesis according to Ghosh et al. [8].

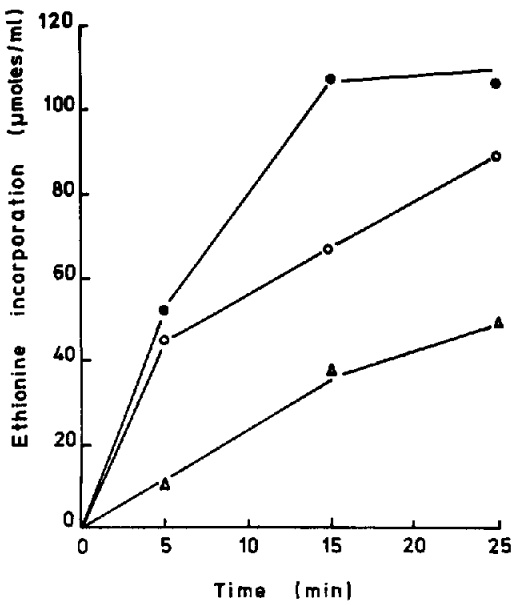

Fig.2. Rate of incorporation of ethionine from [^{14}C]Eth-$tRNA_m^{Met}$ into polyethionine as directed by poly AUG. Reaction mixtures were the same as in table 2. The step two reaction mixture contained either f Eth-$tRNA_f^{Met}$ (•), f Met-$tRNA_f^{Met}$ (○) or neither (△). Samples were withdrawn after 5, 15 and 25 min incubations and assayed as described in table 2.

by the results presented in fig.2 which show that the rate of polyethionine synthesis was slightly greater when initiated by ethionine as compared to methionine.

4. Discussion

The kinetic parameters for the aminoacylation reaction show that ethionine is recognised by methionyl-tRNA synthetase both as a substrate and a competetive inhibitor. The higher K_M values for ethionine indicate that it binds to the enzyme with a lower affinity than methionine. In contrast to the binding of methionine to the enzyme, the binding of ethionine appears to be affected by the type of tRNA present at the reactive site. The K_M for ethionine with $tRNA_m^{Met}$ is higher than that with $tRNA_f^{Met}$ showing that ethionine binds less readily in the presence of $tRNA_m^{Met}$. However once ethionine has bound to the enzyme it is attached to either tRNA at almost the same rate as methionine as the maximum velocities for ethionine are similar to those for methionine.

In the formylation reaction identical K_M and V_{max} values were obtained for ethionyl-$tRNA_f^{Met}$ and methionyl-$tRNA_f^{Met}$. This indicates that the transformylase cannot distinguish between ethionyl-$tRNA_f^{Met}$ and methionyl-$tRNA_f^{Met}$ at the substrate binding site nor in the formylation reaction mechanism. It is interesting to compare this result with the work of Giegé et al. [11] who showed that several species of mischarged *E. coli* $tRNA_f^{Met}$ with (naturally occurring amino acids) could be formylated with identical K_M values to that for methionyl-$tRNA_f^{Met}$ but with much lower V_{max} values. Thus although the structure of the amino acid covalently linked to $tRNA_f^{Met}$ appears to play no part in the recognition of the aminoacyl-$tRNA_f^{Met}$ by the transformylase enzyme at the substrate binding site, it is important in determining the velocity of the formylation reaction. The similarity in the structure and chemical nature of ethionine to methionine permits the formylation reaction to proceed at the same rate as that observed with methionyl-$tRNA_f^{Met}$.

The results of the experiments on in vitro polypeptide synthesis directed by poly AUG show that ethionine is able to replace methionine in polypeptides and that formyl-ethionyl-$tRNA_f^{Met}$ is slightly better than formyl-methionyl-$tRNA_f^{Met}$ in the initiation of polypeptide synthesis. These results add to the results of Kerwar and Weissbach [3] and Giegé et al. [12] who have shown that formylated mischarged species of *E. coli* $tRNA_f^{Met}$ can initiate protein synthesis.

It would appear therefore that in contrast to the aminoacylation reaction where there is a very specific recognition of methionine by both $tRNA_f^{Met}$ and $tRNA_m^{Met}$, in further stages of protein biosynthesis the discrimination between methionine, other amino acids or methionine analogues linked to $tRNA_f^{Met}$ is less strict. Ethionine is not discriminated against at all in the formylation reaction and in vitro polypeptide synthesis directed by poly AUG the initiation stimulated by formylethionyl-$tRNA_f^{Met}$ is more efficient than that stimulated by a naturally occurring initiator tRNA complex. The reason for this is unclear, although it may be that the formyl-ethionyl-$tRNA_f^{Met}$ is more stable to a cleavage activity such as

that described by Ganoza and Barraclough [13] during the initiation process.

References

[1] Bruton, C. J. and Hartley, B. S. (1968) Biochem. J. 108, 281–288.
[2] Trupin, J., Dickerman, H., Nirenberg, M. and Weissbach, H. (1966) Biochem. Biophys. Res. Comm. 24, 50–55.
[3] Kerwar, S. S. and Weissbach, H. (1970) Arch. Biochem. Biophys. 141, 525–532.
[4] Jay, F. T. and Jones, D. S. (1973) Prep. Biochem. 3, 517–523.
[5] Nishimura, S., Jones, D. S. and Khorana, H. G. (1965) J. Mol. Biol. 13, 302–324.
[6] Rabimowitz, J. C. and Pricer, W. E. Jr. (1962) J. Biol. Chem. 237, 2898–2902.
[7] Huennekens, F. M. (1963) Methods Enzymol. 4, 806–811.
[8] Ghosh, H. P., Söll, D. and Khorana, H. G. (1967) J. Mol. Biol. 25, 275–297.
[9] Old, J. M. and Jones, D. S. (1976) FEBS Lett. 64, 148–151.
[10] Lineweaver, H. and Burk, D. (1934) J. Amer. Chem. Soc. 56, 658–666.
[11] Giegé, R., Ebel, J. P. and Clark, B. F. C. (1973) FEBS Lett. 30, 291–295.
[12] Giegé, R., Ebel, J. P., Springer, M. and Grunberg-Manago, M. (1973) FEBS Lett. 37, 166–169.
[13] Ganoza, M. C. and Barraclough, N. (1975) FEBS Lett. 53, 159–163.