INITIATION OF PROTEIN SYNTHESIS WITH MISCHARGED
tRNA$_f^{Met}$ FROM $E.$ $COLI$

R. GIEGÉ and J.P. EBEL

Institut de Biologie Moléculaire et Cellulaire du CNRS,
Laboratoire de Biochimie, 15 rue Descartes, 67000 Strasbourg, France

and

M. SPRINGER and M. GRUNBERG-MANAGO

Institut de Biologie Physico-chimique,
13 rue Pierre et Marie Curie, 75005 Paris, France

1. Introduction

It has previously been shown that aminoacyl-tRNA synthetases may incorrectly aminoacylate several tRNA's [1]. In particular tRNA$_f^{Met}$, the initiator tRNA in *Escherichia coli*, may be charged with phenylalanine and valine with yeast phenylalanyl- and valyl-tRNA synthetases respectively [2]. It has further been demonstrated that phenylalanyl-tRNA$_f^{Met}$ and valyl-tRNA$_f^{Met}$ formed can be enzymatically formylated in the presence of *E. coli* methionyl-tRNA formyltransferase (EC 2.1.2.9) [3]. The question arises now whether these formylated mischarged tRNA$_f^{Met}$ species can initiate protein synthesis in the *E. coli* system. It is shown in the work reported here that an initiation complex can be formed between the ribosome and the formylated phenylalanyl- and valyl-tRNA$_f^{Met}$ in the presence of poly AUG, poly UG and coliphage R$_{17}$ RNA, as well as with formyl-methionyl-tRNA$_f^{Met}$.

2. Materials

Phenylalanyl- and valyl-tRNA$_f^{Met}$ have been prepared as described previously [2, 3]. tRNA$_f^{Met}$ from *E. coli* K12MO (lot 15290) was a gift from the Oak Ridge National Laboratory. Samples of *E. coli* tRNA$_f^{Met}$ of baker's yeast tRNA^{Phe} [4] and tRNA$_2^{Val}$ [5]

were charged with their normal amino acids under usual aminoacylation conditions [1]. The different aminoacyl-tRNA's (1–5 mg) were separated from ATP and the aminoacylation medium by chromatography through small DEAE-cellulose columns (0.6×5 cm) equilibrated with sodium acetate buffer 2×10^{-3}M, pH 4.5. The columns were eluted stepwise with sodium acetate buffer containing 0.3 and 1 M NaCl.

The chemical formylation procedure [6, 7] of aminoacyl-tRNA's has been used instead of the enzymatic one [3] because the former not only gives better yields but also makes possible the formylation of all aminoacyl-tRNA's tested. The formylation obtained ranged from 75 to 90% as judged from samples subjected to alkaline hydrolysis by NH$_4$OH followed by paper electrophoresis at pH 3.5.

Washed ribosomes were prepared as described previously [8] except that 1.5 M NH$_4$Cl buffer was used, instead of 1 M buffer, to wash the ribosomes; moreover these ribosomes were further washed with 1.5 M buffer on a Diaflo XM-100 membrane. Initiation factor IF 2 was prepared as described previously [8], IF 1 and IF 3 were separated on the same DEAE-Sephadex column as IF 2 and purified on carboxymethyl-cellulose columns. IF 3 was further purified on a phospho-cellulose column [9]. These factors were a gift from T. Godefroy-Colburn and J. Dondon.

Table 1

Ribosomal binding and puromycin reaction of formylated and non-formylated aminoacyl-tRNA$_f^{Met}$ species in presence of poly AUG.

		Initiation complex		Puromycin reaction	
		Initiation factors		Initiation factors	
		−	+	−	+
Met-tRNA$_f^{Met}$	−GTP	2.06	1.01	1.23	0.89
(34 pmoles)	+GTP	1.80	1.38	1.07	1.24
fMet-tRNA$_f^{Met}$	−GTP	0.77	3.04	0.79	4.00
(29 pmoles)	+GTP	0.89	8.15	0.95	10.30
Phe-tRNA$_f^{Met}$	−GTP	0.15	0.26	0.20	0.27
(12 pmoles)	+GTP	0.13	0.20	0.19	0.27
fPhe-tRNA$_f^{Met}$	−GTP	0.37	0.49	0.78	1.22
(22 pmoles)	+GTP	0.39	2.50	0.75	5.20
Val-tRNA$_f^{Met}$	−GTP	4.42	0.93	0.37	0.54
(33 pmoles)	+GTP	4.42	1.56	0.42	0.36
fVal-tRNA$_f^{Met}$	−GTP	1.77	2.00	0.62	1.86
(37 pmoles)	+GTP	1.89	9.75	0.64	10.90

The incubation mixture (50 μl) contains: Tris–HCl pH 7.5, 50mM; NH$_4$Cl, 80mM; magnesium acetate, 5 mM; β-mercaptoethanol, 7mM; ribosomes, 1.72 uA_{260}; GTP, 1 mM; deacylated crude tRNA 1.6 uA_{260}; poly AUG (when indicated), 0.315 uA_{260}; formylated aminoacyl-tRNA's as indicated in the table; IF$_1$, 0.33 μg; IF$_2$, 0.6 μg; IF$_3$, 0.18 μg. Incubation was at 37°C for 20 min. For aminoacyl-tRNA binding determination 3 ml of the following buffer was added to the incubation mixture: Tris–HCl pH 7.5, 50 mM; NH$_4$Cl, 80 mM; magnesium acetate, 5 mM. The samples are then immediately filtered on a Millipore (HAWP 025000) filter. The filters are washed twice with 3 ml of the same buffer, dried and counted in an Intertechnique scintillation counter. For the puromycin reaction 100 μl of a solution of the antibiotic (puromycin dihydrochloride, 0.7 mg/ml; Tris–HCl pH 7.5 50 mM; NH$_4$Cl, 80 mM; magnesium acetate, 5 mM; β-mercaptoethanol, 7 mM) was added to each sample after 20 min-incubation in the same conditions as in the binding determination. Incubation was continued for 5 min at 37°C and then the sample treated as already published [13, 14] and counted in the same scintillation counter. Results are expressed in picomoles of (formyl)-aminoacyl-tRNA$_f^{Met}$ bound to ribosomes or in picomoles of (formyl)-aminoacyl-puromycin.

Poly UG and poly AUG were generously given by M. N. Thang.

RNA from phage R$_{17}$ was prepared according to Gesteland and Boedtker [10] except that E. coli strain JC 1553 KLF 41 was used to grow the phage. The origin of other products used is as follows: total tRNA from E. coli , Schwarz, USA; puromycin, NBC, USA; 14C-labelled amino acids, C.E.A., France.

3. Results

3.1 Binding reactions

We first studied the binding to ribosomes of the various species of formylated and non-formylated aminoacyl-tRNA$_f^{Met}$ in the presence of the synthetic poly-nucleotide poly AUG. The conditions used for these experiments allow the formation of a functional initiation complex where formyl-methionyl-tRNA$_f^{Met}$ is bound at the P site and thus can react with puromycin at low magnesium concentration [11, 12].

Table 1 indicates that under these conditions the binding of formylated methionyl-tRNA$_f^{Met}$ is dependent upon initiation factor and GTP addition. The binding of the mischarged formylated tRNA$_f^{Met}$ species is stimulated approx. 5-fold by initiation factors in the presence of GTP, while in the absence of GTP no strong stimulation is found.

Similar results were found using poly UG instead of poly AUG as messenger RNA.

As a control, in experiments not reported in table 1 we repeated the binding reactions with formyl-

Table 2

Ribosomal binding and puromycin reaction of valy- and formyl-valyl-tRNA$_f^{Met}$ in presence of R_{17} RNA.

	R_{17} RNA	Initiation complex				Puromycin reaction			
		Initiation factors				Initiation factors			
		−		+		−		+	
		−GTP	+GTP	−GTP	+GTP	−GTP	+GTP	−GTP	+GTP
Val-tRNA$_f^{Met}$	−	0.20	0.12	0.13	0.12	0.46	0.43	0.44	0.43
(33 pmoles)	+	0.17	0.10	0.13	0.12	0.48	0.49	0.45	0.49
fVal-tRNA$_f^{Met}$	−	0.23	0.27	0.24	0.53	0.21	0.19	0.23	0.58
(37 pmoles)	+	0.21	0.22	0.22	0.82	0.20	0.19	0.23	1.70

The conditions are the same as in table 1. 0.7 uA_{260} of R_{17} RNA was used instead of Poly AUG. Results are expressed in picomoles of (formyl)-valyl-tRNA$_f^{Met}$ bound to ribosomes or in picomoles of (formyl)-valyl-puromycin.

aminoacyl-tRNA species other than methionyl-tRNA$_f^{Met}$ in which the tRNA moiety corresponds to the amino acid. Under the condition described above, binding of formyl-valyl-tRNA^{Val} was not increased by initiation factors, whereas that of formyl-phenylalanyl-tRNA^{Phe} was slightly increased.

Also shown in table 1 is that the binding of the non-formylated mischarged tRNA$_f^{Met}$ species is lower than observed for the formylated species. The binding of both phenylalanyl- and valyl-tRNA$_f^{Met}$ is insensitive to GTP. Our data indicate only a low residual binding of phenylalanyl-tRNA$_f^{Met}$ which is not stimulated by the initiation factors. On the contrary, the binding of valy-tRNA$_f^{Met}$, in absence of initiation factors, has been found relatively strong. This binding is inhibited by initiation factors. Similarly, the weaker binding of methionyl-tRNA$_f^{Met}$ is also inhibited by initiation factors.

Table 2 shows the same experiments performed with R_{17} RNA. The results are identical to those obtained with poly AUG except that we did not find the relatively strong binding with valyl-tRNA$_f^{Met}$ in the absence of initiation factors.

3.2 Puromycin reactions

In order to demonstrate that the formyl-phenyla-lanyl-tRNA$_f^{Met}$ and the formyl-valyl-tRNA$_f^{Met}$ are correctly accomodated on the ribosome we measured the reaction with puromycin of the various formylated

aminoacyl-tRNA$_f^{Met}$ species. Formyl-methionyl-tRNA$_f^{Met}$ and peptidyl-tRNA's are known to react with puromycin only when bound at the P site of the ribosome [13]. Our results indicate that the formylated mischarged tRNA$_f^{Met}$ species react with puromycin as well as formyl-methionyl-tRNA$_f^{Met}$ does. All these reactions are GTP and initiation factor dependent.

In control experiments we did not find any reaction between puromycin and the non-formylated aminoacyl-tRNA$_f^{Met}$ species.

Identical results were found using R_{17} RNA (table 2) instead of poly AUG for the puromycin reaction.

4. Discussion

The experiments reported here show that formyl-valyl-tRNA$_f^{Met}$ and formyl-phenylalanyl-tRNA$_f^{Met}$ behave similarly to formyl-methionyl-tRNA$_f^{Met}$ during the initiation step of protein synthesis. This was proved (i) by initiation factor and GTP dependent binding of the formylated mischarged tRNA$_f^{Met}$ species to the ribosomes and (ii) by reactivity with puromycin. These reactions occur in the same way with poly AUG and poly UG, showing therefore that the incorrectly acylated tRNA$_f^{Met}$ species respond to the two codons AUG and GUG.

Moreover we found that the non-formylated valyl-tRNA$_f^{Met}$ forms a poly AUG dependent complex with

the ribosomes, but this complex does not respond to GTP, is inhibited by initiation factors and does not react with puromycin. This suggests that valyl-tRNA$_f^{Met}$, in contrast with the formylated products, is bound on the ribosomes in a way not allowing protein synthesis. This particular behaviour of valyl-tRNA$_f^{Met}$ is not observed when R_{17} RNA is used as a message.

Concerning the requirements for a correct initiation complex our results suggest that the structure of tRNA itself is essential for the specificity of the initiation step. On the contrary, the nature of the amino acid bound to the initiator tRNA seems not to be important, for the correct construction of the initiation complex, as the replacement of the methionine moiety in the formylated methionyl-tRNA$_f^{Met}$ by phenylalanine or valine does not change the specific properties of this tRNA during initiation. Moreover the formylation of the wrong amino acids linked to tRNA$_f^{Met}$ is necessary to observe puromycin reactive initiation complexes.

The results of Kerwar and Weissbach in vitro [15] and of Brown in vivo [16] show that initiation is possible when methionine is replaced by its analogues ethionine and norleucine. In the work reported here we emphasize that methionine may also be replaced in vitro by structurally unrelated amino acids such as phenylalanine or valine.

Acknowledgments

References

[1] Giegé, R., Kern, D. and Ebel, J.P. (1972) Biochimie 54, 1245–1255.
[2] Kern, D., Giegé, R. and Ebel, J.P. (1972) Eur. J. Biochem. 31, 148–155.
[3] Giegé, R., Ebel, J.P. and Clark, B.F.C. (1973) FEBS Letters 30, 291–295.
[4] Dirheimer, G. and Ebel, J.P. (1967) Bull. Soc. Chim. Biol. 49, 1679–1687.
[5] Bonnet, J., Dirheimer, G. and Ebel, J.P. (1969) Bull. Soc. Chim. Biol. 51, 415–418.
[6] Rudland, P.S., Whybrow, W.A., Marcker, K.A. and Clark, B.F.C. (1969) Nature 222, 750–753.
[7] Gillam, I., Blew, D., Warrington, R.C., Von Tigerstrom, M. and Tener, G.M. (1968) Biochemistry 7, 3459–3468.
[8] Lelong, J.C., Grunberg-Manago, M., Dondon, J., Gros, D. and Gros, F. (1970) Nature 226, 505–510.
[9] Sabol, S., Meier, D. and Ochoa, S. (1973) Eur. J. Biochem. 33, 332–340.
[10] Gesteland, R.F. and Boedker, H. (1964) J. Mol. Biol. 8, 496–507.
[11] Springer, M., Dondon, J., Graffe, M., Grunberg-Manago, M., Lelong, J.C. and Gros, F. (1971) Biochimie 53, 1047–1057.
[12] Springer, M. and Grunberg-Manago, M. (1972) Biochem. Biophys. Res. Commun. 47, 477–484.
[13] Traut, R.R. and Monro, R.E. (1964) J. Mol. Biol. 10, 63–72.
[14] Leder, P. and Bursztyn, H. (1966) Biochem. Biophys. Res. Commun. 25, 233–238.
[15] Kerwar, S.S. and Weissbach, H. (1970) Archiv. Biochem. Biophys. 141, 525–532.
[16] Brown, J.L. (1973) Biochim. Biophys. Acta 294, 527–529.