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FORMYLATION OF MISCHARGED *E. COLI* tRNA$_f^{Met}$

R. GIEGÉ and J.P. EBEL
Laboratoire de Chimie Biologique, Université Louis Pasteur,
Rue Descartes, Esplanade, 67000 Strasbourg, France

and

B.F.C. CLARK
M.R.C. Laboratory of Molecular Biology,
Hills Road, Cambridge CB2 2QH, England

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1. Introduction

In a previous work we demonstrated the mischarging of *Escherichia coli* tRNA$_f^{Met}$ by yeast phenylalanyl- and valyl-tRNA synthetases [1]. The possibility of obtaining phenylalanyl-tRNA$_f^{Met}$ and valyl-tRNA$_f^{Met}$ allows the study of the properties of these mischarged species of initiator tRNA during the different steps of the initiation process of protein synthesis. The first reaction of the initiation mechanism in *E. coli* is the formylation of methionyl-tRNA$_f^{Met}$. It was therefore necessary first to verify if an incorrectly aminoacylated initiator tRNA is able to be formylated. In the present paper we demonstrate that either phenylalanyl-tRNA$_f^{Met}$ or valyl-tRNA$_f^{Met}$ can be formylated *in the presence* of the transformylase from *E. coli*. These results suggest that the specificity of the formylation reaction exclusively depends upon the nature of the tRNA moiety of the aminoacylated tRNA$_f^{Met}$ and not upon that of the amino acid bound to the tRNA.

2. Materials

tRNA$_f^{Met}$ from *E. coli* K12 MO (lot 15–290) was a gift from the Oak Ridge National Laboratory.

Highly purified yeast phenylalanyl-tRNA synthetase (PheRS) [2] and valyl-tRNA synthetase (ValRS) [3] were gifts from F. Fasiolo and D. Kern (Strasbourg). Pure *E. coli* methionyl-tRNA synthetase (MetRS) prepared according to Bruton and Hartley [4], was a gift from Dr. C.J. Bruton (Cambridge). A crude extract from *E. coli*, devoid of nucleic acids but still containing the aminoacyl-tRNA synthetases, was used as a transformylase preparation [5].

An acidic solution of radioactive N_{10}[^{14}C]formyltetrahydrofolic acid (50 mCi/mmole) was a kind gift from Dr. G. Koch (Cambridge) (the concentration of formyltetrahydrofolic acid in that solution could not be precisely estimated). Cold N_{10}-formyltetrahydrofolic acid was prepared according to Jones et al. [6]. L-[^{14}C]methionine (200 mCi/mmole) was from NEN Chemicals GmbH. L-[^{14}C]phenylalanine (492 mCi/mmole) and L-[^{14}C]valine (260 mCi/mmole) were from the Radiochemical Centre, Amersham. [^{14}C] labelled *N*-formylamino acids have been prepared according to Sheenan and Yang [7]. Bovine pancreatic ribonuclease A (type IA) was from Sigma. All other chemicals were of the highest purity available commercially.

Kodak "Rapid Processing" (RP/S 14) X-ray films were employed for autoradiography.

3. Methods and results

3.1. *Different aminoacylation conditions of E. coli tRNA$_f^{Met}$*

Normal aminoacylation of *E. coli* tRNA$_f^{Met}$ (5 μg per 100 μl incubation mixture) by methionine was conducted in a medium containing 50 mM Tris-HCl pH 7.8, 50 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM ATP, 7 mM β-mercaptoethanol, 10 μM methionine (^{12}C or ^{14}C-labelled: 200 mCi/mmole) and 0.5 μg purified MetRS per 5 μg $tRNA_f^{Met}$. The incubation was 15 min at 37°. In these conditions the methionine acceptance was about 1600 pmoles per A_{260} unit.

Incorrect phenylalanine charging on *E. coli* $tRNA_f^{Met}$ was performed in special experimental conditions as described by Kern et al. [1]; in particular the Mg^{2+}/ATP ratio was 15 and the medium contained 20% of dimethylsulfoxide, 10 μM phenylalanine (^{12}C or ^{14}C-labelled: 250 mCi/mmole) and 5 μg purified yeast PheRS per 5 μg $tRNA_f^{Met}$. Under these conditions, 40 to 60% of the $tRNA_f^{Met}$ molecules could be loaded with phenylalanine.

Valine was attached to purified *E. coli* $tRNA_f^{Met}$ using the following *in vitro* conditions [8]: 50 mM Hepes-KOH pH 7.6, 3 mM $MgCl_2$, 1 mM ATP (Mg^{2+}/ATP = 3), 1 mM dithioerythrytol, 10 μM valine (^{12}C or ^{14}C labelled: 260 mCi/mmole) and 4.5 μg purified yeast ValRS per 7.5 μg $tRNA_f^{Met}$ per 100 μl incubation mixture. The incubation was 3 hr at 30°. These conditions lead to a complete aminoacylation of the purified $tRNA_f^{Met}$ and have therefore been preferred to those used in a previous work [1] which led to only a partial mischarging reaction.

3.2. *Formylation conditions*

The formylation reactions were conducted in the following medium: 50 mM Tris-pH 7.8, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 50 mM NH_4Cl, 3 to 5 μg aminoacyl-$tRNA_f^{Met}$ per 100 μl and appropriate quantities of formyltetrahydrofolic acid and of enzyme. For the reactions performed in the presence of radioactive formyltetrahydrofolic acid, we previously determined the optimal quantity of product needed to get optimal formylation: generally we used 10 μl of the radioactive compound per 100 μl of the formylation medium. In the case of reactions performed in the presence of cold formyltetrahydrofolate, it was necessary before use to reduce the folate. This was performed in the following way: a few mg of formyltetrahydrofolate powder were dissolved in 1 ml of 50 mM phosphate buffer pH 7.5 and this mixture was then subjected to hydrogenisation by a flow of gaseous hydrogen during 2 hr at room temp. in the presence of a "5% Rhodium-alumina" catalyst. At the end of the reaction the catalyst was spun down and 5 μl samples of the supernatant were used per 100 μl of formylation medium. Further experimental details are given in the legends of table 1 and of fig. 1.

Table 1
Formylation of various species of aminoacylated $tRNA_f^{Met}$ from *E. coli*

	Aminoacyl-$tRNA_f^{Met}$		
	Methionyl-	Phenylalanyl-	Valyl-
Percentage of aminoacylation	100	60	100
Optimal incorporation of [^{14}C]formyl groups in the aminoacylated $tRNA_f^{Met}$ (cpm/test)	3425^a	2223^a	809^b
Calculated percentage of formylation of the aminoacyl-$tRNA_f^{Met}$	82	90	20

Samples of 100 μg *E. coli* $tRNA_f^{Met}$ have been loaded with cold methionine, phenylalanine or valine as described in sect. 3.1 and the tRNA's recovered from the aminoacylation media after phenol extraction and ethanol precipitation. (The aminoacylation extents have been estimated in a parallel experiment using ^{14}C-labelled amino acids). Then the charged $tRNA_f^{Met}$'s (5 μg) have been incubated at 30° in 200 μl of the formylation medium containing a large excess of a crude *E. coli* enzyme extract (300 μg proteins) and 10 μl of [^{14}C]formyltetrahydrofolic acid. The radioactivity of 50 μl samples was measured on Millipore filter discs after 10, 20 and 30 min. Appropriate controls, without tRNA or without aminoacylated tRNA's have been performed. Results corresponding to the optimal incorporation of radioactivity in the aminoacylated $tRNA_f^{Met}$ (expressed in cpm per 50 μl of incubation mixture) are reported. Similar results have been obtained by adding the transformylase and [^{14}C]formyltetrahydrofolic acid directly to the different aminoacylation media after the end of the aminoacylation reactions. (It must however be noticed that in that case we have adjusted the pH, the β-mercaptoethanol and the magnesium concentration to optimal values for the formylation reaction).

^{a} Result obtained after 10 min of incubation; ^{b} after 30 min.

3.3. *Formylation of various species of aminoacyl-$tRNA_f^{Met}$*

The first evidence, showing that various species of *E. coli* aminoacyl-$tRNA_f^{Met}$ can be formylated by the *E. coli* transformylase is shown in table 1, where the

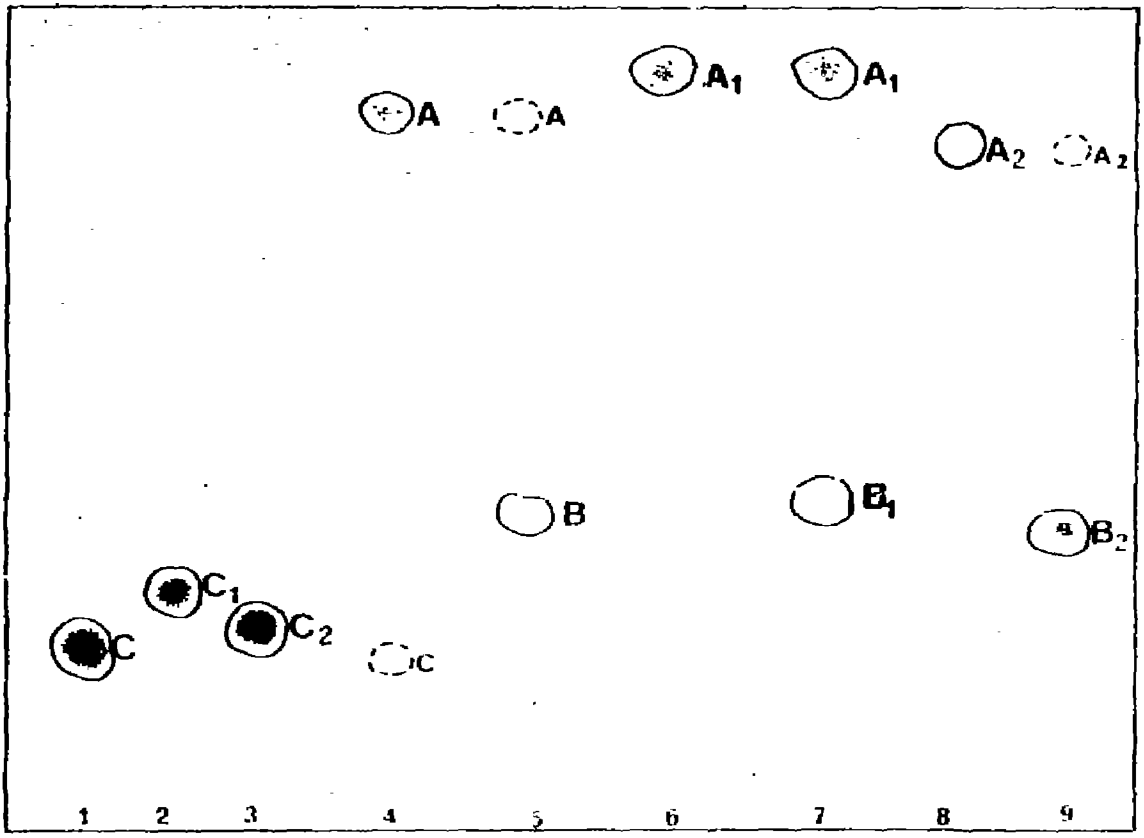

Fig. 1. Radioautographs arising from pancreatic ribonuclease digests of various species of [^{14}C]aminoacyl-tRNA$_f^{Met}$ subjected or not to formylation conditions. 10 μg of aminoacylated tRNA$_f^{Met}$ (corresponding to approx. 0.1 μCi of methionyl- or valyl- or to 0.05 μCi of phenylalanyl-tRNA$_f^{Met}$) were incubated during 15 min at 37° in 200 μl of formylation medium containing an excess (300 μg) of *E. coli* enzyme preparation. Reactions were stopped by adding 10 μl of 2 M sodium acetate pH 5.2. 200 μg carrier tRNA were then added and the tRNA's were immediately recovered by phenol extraction and ethanol precipitation. The precipitates were washed twice with a solution of ethanol containing 0.1 M acetate pH 5.2 (3:2, v/v) and lyophilised. The tRNA residues were dissolved in 10 μl water and 5 μl of these tRNA mixtures were incubated during 5 min at 37° in the presence of 5 μl of a solution of pancreatic ribonuclease (1 mg per ml of 0.01 M MES buffer pH 6.0). These 10 μl samples of the ribonuclease digestions were fractionated by electrophoresis according to Marcker and Sanger [9] and electrophoretograms were autoradiographed during 12 hr. Spots A, A_1 and A_2 correspond respectively to methionyl-, valyl- and phenylalanyl-adenosine; spots B, B_1 and B_2 to formylmethionyl-, formylvalyl- and formylphenylalanyl-adenosine and spots C, C_1 and C_2 to the amino acids methionine, valine and phenylalanine. Experiments 1, 2 and 3 correspond to the electrophoretic migration of the control amino acids (○ represents the starting point of migration), experiments 4, 6 and 8 correspond to control hydrolysates of methionyl-, valyl- and phenylalanyl-tRNA$_f^{Met}$ which were not subjected to the formylation conditions and experiments 5, 7 and 9 correspond to the hydrolysates of formylmethionyl-, formylvalyl- and formylphenylalanyl-tRNA$_f^{Met}$.

incorporation of radioactive [^{14}C]formyl groups into tRNA$_f^{Met}$ charged with cold amino acids is reported. A rough calculation from the data of these experiments indicates that the formylation of methionyl- and phenylalanyl-tRNA$_f^{Met}$ seems to be much easier than that of valyl-tRNA$_f^{Met}$. However, these experiments do not rigorously demonstrate, in the case of the mischarged tRNA$_f^{Met}$, if the formyl groups are incorporated either onto the $-NH_2$ moiety of the amino acids attached on tRNA$_f^{Met}$ or on another part of the aminoacylated tRNA$_f^{Met}$. The last possibility is however unlikely as non-aminoacylated tRNA$_f^{Met}$ is unable to be labelled by radioactive [^{14}C]formyltetrahydrofolic acid in our experimental conditions.

In order to confirm the results of table 1 and to characterise the formylated amino acids, we studied in further experiments the formylation reaction using labelled species of [^{14}C]aminoacyl-tRNA$_f^{Met}$ as sub-

Table 2
Kinetic parameters of the formylation reactions.

	Aminoacyl-tRNA$_f^{Met}$		
	Methionyl-	Phenylalanyl-	Valyl-
V_{max} (arbitrary units)	57	19	1
K_m (μM)	0.6	1.3	1.4

The formylation of the different species of aminoacyl-tRNA$_f^{Met}$ was measured in the presence of radioactive formyltetrahydrofolic acid.

strates for the transformylase. The pancreatic ribonuclease digests of these charged species of tRNA$_f^{Met}$, previously incubated in the formylation mixture, were analysed, according to Marcker and Sanger [9], by electrophoresis on Whatmann 3MM paper at pH 3.5 (3000 V during 1 hr) (the experimental details are given in the legend of fig. 1). The results of fig. 1 indicate first that the classical formylation of the methionyl-tRNA$_f^{Met}$ is complete as expected from previous experiments [10, 11]; indeed a single spot (B), which is characteristic for an N-blocked methionyl-adenosine, was found on the radioautograph. Concerning the mischarged tRNA$_f^{Met}$, our results show also that formylation takes place either on phenylalanine or on valine when they are bound to initiator tRNA. In these cases radioactive spots (B_1 and B_2) are found, which have a reduced mobility toward the cathode and which do not correspond to the free amino acids migrating as C_1 and C_2. We demonstrated that these spots correspond to the formylaminoacyl-adenosines. This was shown using the method described by Marcker and Sanger [9]. In this way we characterised i) the formylvaline and the formylphenylalanine obtained after an alkali treatment of the material eluted from spots B_1 and B_2 by comparison with reference compounds and ii) the free radioactive valine and phenylalanine liberated after a further acidic hydrolysis of these formylamino acids.

3.4. *Study of the kinetic parameters of the formylation reactions*

The experiments reported in fig. 1, as well as those reported in table 1, in which the formylation of the mischarged species of tRNA$_f^{Met}$ is demonstrated, however suggest some differences in the reactivities of these aminoacyl-tRNA's towards the transformylase. For instance the formylation of phenylalanyl-tRNA$_f^{Met}$ is practically complete, as only a trace spot (A_2) corresponding to phenylalanyl-adenosine has been found, whereas that of valyl-tRNA$_f^{Met}$ seems to be only partial, at least under the experimental conditions used, even in the presence of large quantities of enzyme preparation. Indeed in the latter case we have detected on the radioautograph two major spots (A_1 and B_1: experiment no. 7) corresponding respectively to valyl-adenosine and to formylvalyl-adenosine. A counting of the radioactivity of these spots indicates, in good agreement with the results of table 1, a formylation of 20% of the molecules of valine bound to tRNA$_f^{Met}$.

In order to obtain more precise information concerning this problem of the reactivity of the various species of aminoacyl-tRNA$_f^{Met}$ in the transformylation reaction, we have studied their kinetic parameters. In table 2, the V_{max} and K_m values of the classical formylation reaction involving methionyl-tRNA$_f^{Met}$ and that of the non-classical reactions involving valyl- or phenylalanyl-tRNA$_f^{Met}$ are compared. It appears that the differences in reactivity observed are especially linked to changes in the velocity of the formylation reaction. Indeed the V_{max} of the formylation reaction of valyl-tRNA$_f^{Met}$ was found 60-fold reduced and that corresponding to the formylation of phenylalanyl-tRNA$_f^{Met}$ only 3-fold, as compared to that of the classical formylation of methionyl-tRNA$_f^{Met}$. As far as the K_m's are concerned nearly identical values have been found. These values must however, especially in the case of phenylalanyl-tRNA$_f^{Met}$, be considered as rough estimates, as non-aminoacylated tRNA$_f^{Met}$, acting as a competitive inhibitor in the formylation reaction, is present in the incubation mixture, leading therefore to apparent K_m values. The value found for methionyl-tRNA$_f^{Met}$ differs by a factor of 10 (10 μM) from that given by Dickerman et al. [12]. Differences in the assay conditions as well as in the purity of the tRNA$_f^{Met}$ used, or in their extent of aminoacylation could explain the discrepancy between this result and ours.

4. Discussion

Since the discovery of the enzymatic formylation of methionyl-tRNA$_f^{Met}$ from *E. coli* [9], relatively little has been found about the structural requirements of methionyl-tRNA$_f^{Met}$ responsible for the specificity of the formylation reaction. It is well established that the structure of tRNA$_f^{Met}$ itself is one of the parameters responsible for this specificity [10, 11]. In contrast it is still uncertain whether the structure of methionine plays a role in specifying the formylation. The only information about this point comes from the experiments demonstrating the formylation of norleucyl-tRNA$_f^{Met}$ [13, 14], but this result cannot be considered as evidence for the non-involvement of the aminoacyl moiety in the specificity of the formylation reaction, since norleucine is a structural analog of methionine. The chief reason for this lack of information has been the impossibility, until recently, of obtaining a mischarged tRNA$_f^{Met}$. Our previous work on mischarging [1], and the results described in this paper, demonstrating the formylation of either phenylalanyl- or valyl-tRNA$_f^{Met}$ from *E. coli*, allow us to answer this question. Indeed, the possibility of formylating tRNA$_f^{Met}$, mischarged with amino acids structurally unrelated with methionine, implies that the aminoacyl moiety is not primarily involved in the recognition mechanism between aminoacyl-tRNA$_f^{Met}$ and the transformylase. This idea is also supported by indirect evidence, coming from experiments studying protein synthesis in the presence of isoleucyl-tRNA$_f^{Met}$, which suggest a possible formylation of isoleucine bound to tRNA$_f^{Met}$ [15]. It appears therefore that the specificity of the formylation reaction is exclusively controlled by the nature of the tRNA moiety of the aminoacylated tRNA$_f^{Met}$ and not by the amino acid bound to the tRNA. Thus it can be postulated that the affinities between the various species of aminoacyl-tRNA$_f^{Met}$ and the transformylase must be identical. We have actually confirmed this prediction as we have found identical K_m values for the formylation of methionyl-, phenylalanyl- and valyl-tRNA$_f^{Met}$.

Although our experiments clearly demonstrate that the nature of the amino acid bound to tRNA$_f^{Met}$ is unimportant in the specificity of the formylation reaction, they do not exclude an involvement of this bound amino acid on a level other than that of the recognition. Indeed we have observed reduced velocities for the formylation when the tRNA$_f^{Met}$ has been mischarged with phenylalanine and especially with valine. Consequently it may be that the chemical nature of the amino acid can play a part during the catalysis by allowing a more or less good fit of the aminoacyl $-NH_2$ group in the catalytic site of the enzyme.

Another useful result of this work is the possibility of it leading to further experiments in the field of initiation of the protein synthesis, using various species of formylated mischarged tRNA$_f^{Met}$. The results presented in this paper are prerequisites for such investigations. Current work is proceeding in that direction.

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