PEPTIDE CHAIN INITIATION WITH CHEMICALLY FORMYLATED MET-tRNAs FROM E. COLI AND YEAST

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Received February 19,1973

SUMMARY: Chemically formylated Met-$tRNA_m^{Met}$ and Met-$tRNA_f^{Met}$ species from E. coli and yeast were tested for their capacity to serve as chain-initiators in a cell-free system from E. coli. In the presence of R 17 mRNA, initiation factors and E. coli ribosomes, all four Met-tRNAs could form functional initiation complexes as measured by ribosomal binding kinetics, fMet-puromycin formation and synthesis of a dipeptide fMet-Ala. Unformylated Met-$tRNA_f^{Met}$ from E. coli displayed significantly less activity as a peptide chain-initiator than the formylated Met-$tRNA_m^{Met}$ species from E. coli and yeast. Although the latter tRNAs were less effective initiators than the "physiological" initiator tRNAs, the data seem to indicate that a blocked α-amino group represents the major token of identification by which Met-tRNA is admitted to function in E. coli peptide chain initiation.

INTRODUCTION: Bacteria as well as eucaryotic cells contain two types of methionine-accepting tRNAs: $tRNA_f^{Met}$ and $tRNA_m^{Met}$ (1 - 3). In bacteria, the functional restriction of each of these two $tRNA^{Met}$ species to either chain initiation or elongation is secured by two mechanisms one of which operates at the level of N-formylation for which only Met-$tRNA_f^{Met}$ is a suitable substrate while the other is active at the level of translation where each of the two Met-$tRNA^{Met}$ species is recognized by specific factors.

In this paper an attempt was made to delineate the relative importance of N-formylation on one hand and structural features inherent in the tRNA itself on the other hand as control parameters for the function of a given Met-tRNA in chain initiation. It is shown that chemically formylated Met-$tRNA_m^{Met}$ species from E. coli and yeast can initiate new peptide chains in cell-free systems from E. coli when a natural mRNA and initiation factors are present whereas unformylated initiator tRNA from E. coli is a very poor substrate for peptide chain initiation under these conditions. The results emphasize the functional significance of the formyl-methionyl moiety in making a $tRNA^{Met}$ molecule acceptable for the initiation machinery.

EXPERIMENTAL: The procedures for the separation and purification of the iso-accepting $tRNA^{Met}$ species from E. coli and yeast were described previously (4, 5). Pure $tRNA_f^{Met}$ (E. coli) was purchased from Boehringer, Mannheim, Germany. Aminoacylation and formylation assays were carried out according to Doctor et al. (6). $tRNA^{Ala}$ (E. coli) was prepared from total E. coli-tRNA by reversed phase chromatography no. 6 (7). All tRNAs were charged with homologous enzyme preparations as described previously (5, 8). Chemical formylation of all Met-$tRNA^{Met}$ species was performed using N-formyloxy-succinimide the procedure for formylation being essentially that of Gillam et al. (9). Acetic-formic anhydride was prepared as described by Fieser and Fieser (10). Treatment of $tRNA_f^{Met}$ (E. coli) with acetic-formic anhydride was analogous to the procedure of Haenni and Chapeville (11). E. coli "run off"-ribosomes were washed three times in standard buffer containing 0.5 M NH_4Cl. The wash was used as a source for crude initiation factors as described by Revel et al. (12). Factor $T_{u,\,s}$ was prepared according to Gordon (13). The growth of phage R 17, its purification and the isolation of R 17 RNA followed standard procedures (14). Digests of $tRNA_f^{Met}$ (E. coli) with ribonuclease T_1 were prepared and chromatographed as reported by Seno et al. (15). The ribosomal binding assays were carried out at 30° C in 0.1 ml volumes containing 2.1 A_{260} units of ribosomes, 20 pmoles of ^{35}S-labeled fMet-tRNA, 0.92 units of R 17 RNA, 130 mM KCl, 15 mM Tris-HCl, pH 7.5, 5 mM Mg-acetate, 1 mM glutathione, 0.2 mM GTP, and 35 µg of initiation factor protein. Reactions were terminated by dilution with buffer and immediate filtration on nitrocellulose filters. The formation of fMet-puromycin was assayed according to Leder and Bursztyn (16), the identity of the product was ascertained by high-voltage electrophoresis (8). For the synthesis of the dipeptide fMet-Ala, binding of fMet-tRNA to ribosomes was allowed to proceed for 40 minutes. Subsequently, 20 pmoles of [^3H]Ala-$tRNA^{Ala}$ (E. coli) and 18 µg of factor $T_{u,\,s}$ were added and incubations continued for another 5 minutes. The reactions were terminated as described above. After the

radioactivity had been measured, the dipeptide and unreacted amino acids were hydrolyzed off the tRNA bound to the filters with NH_4OH (pH 10.6). The extracts were brought to dryness and separated by high-voltage electrophoresis at 16 V/cm in pyridine acetate buffer pH 4.7 on Whatman 3 MM paper. After the run, the papers were dried and cut into strips of 0.5 cm width for liquid scintillation counting. The positions of standard fMet and fMet-Ala were located by color reactions (17).

RESULTS: Fig. 1 illustrates the time dependent formation of R 17 coat protein

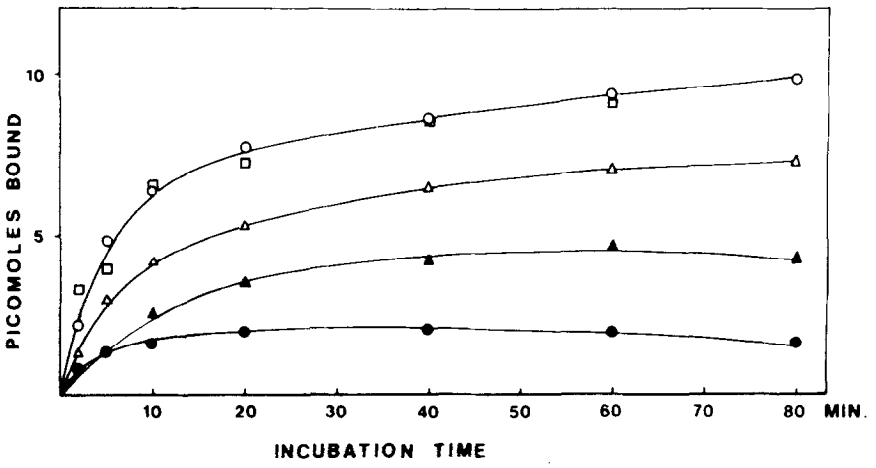

Fig. 1: Binding of chemically formylated [^{35}S]Met-tRNA species from E. coli and yeast to E. coli ribosomes as a function of time. Open circles: fMet-$tRNA_f^{Met}$ (E. coli), closed circles: fMet-$tRNA_m^{Met}$ (E. coli), open triangles: fMet-$tRNA_f^{Met}$ (yeast), closed triangles: fMet-$tRNA_m^{Met}$ (yeast), all chemically formylated. Open squares: values obtained with enzymatically formylated fMet-$tRNA_f^{Met}$ (E. coli).

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initiation complexes with fMet-$tRNA_f^{Met}$ and fMet-$tRNA_m^{Met}$ from E. coli and the two corresponding fMet-tRNA species from yeast. The binding curve of fMet-$tRNA_f^{Met}$ from yeast parallels that of E. coli initiator tRNA. Interestingly, significant attachment of the fMet-$tRNA_m^{Met}$ species from yeast and E. coli is also observed. However, while the binding of the initiator tRNAs has not quite reached saturation after 80 minutes of incubation the binding of

fMet-tRNA$_m^{Met}$ from E. coli starts to decline after 40 minutes of incubation, that of fMet-tRNA$_m^{Met}$ (yeast) after 60 minutes. As shown in table 1, the

Table 1

Binding of f[^{35}S]Met-tRNAs from E. coli and yeast to E. coli ribosomes and formation of fMet-puromycin.

[^{35}S]Met-tRNA	IF[1)]	R 17 RNA	picomoles bound	picomoles reacted with puromycin	% fMet converted
Met-tRNA$_f^{Met}$ (E. coli)	-	+	0.07	0.32	
	+	+	0.23	0.58	--
fMet-tRNA$_f^{Met}$ (E. coli)	-	+	0.10	0.61	
	+	+	7.95	7.24	91
fMet-tRNA$_m^{Met}$ (E. coli)	-	+	0.14	0.15	
	+	+	2.10	1.39	66
fMet-tRNA$_f^{Met}$ (yeast)	-	+	0.08	0.35	
	+	+	5.70	4.97	87
fMet-tRNA$_m^{Met}$ (yeast)	-	+	0.14	0.13	
	+	+	3.80	4.21	110

20 pmoles of each f[^{35}S]Met-tRNA were incubated with 2.1 A_{260} units of ribosomes, 0.92 A_{260} units of R 17 RNA, 35 µg of initiation factors and other constituents as described in the text. The amount of each fMet-tRNA bound to ribosomes after 25 min. was determined. Puromycin was added to one set of tubes after 5 min. of incubation. fMet-puromycin formed during a subsequent incubation period of 20min. was extracted with 2 ml of ethylacetate at pH 8.0 and identified by high voltage electrophoresis. All values represent averages from two experiments, each carried out in duplicate. No radioactive material could be extracted from control tubes incubated without ribosomes but with all other constituents.

binding of each of the four fMet-tRNAs is fully sensitive to puromycin. Accordingly, one should expect these fMet-tRNAs to be capable of initiating the formation of NH_2-terminal dipeptide of the R 17 coat protein. This is indeed the case: when, after 40 min. of initiation complex formation with the f[^{35}S]Met-tRNA species from E. coli and yeast, [^{3}H]Ala-tRNA^{Ala} is added to the reaction mixtures and the incubation continued for 5 min. virtually all formyl-methionine bound is incorporated into the dipeptide fMet-Ala (table 2). As in the two experiments described above, no functional difference between the chemically and the enzymatically formylated fMet-tRNA$_f^{Met}$ species from

Table 2

Formation of fMet-Ala in response to R 17 mRNA.

[^{35}S]Met-tRNA	bound to ribosomes (picomoles) Met-tRNA	Ala-tRNA	dipeptide formed (pmoles)	% of bound Met-tRNA
fMet-tRNA$_f^{Met}$ (E. coli)[2]	6.80	7.15	5.64	83
fMet-tRNA$_f^{Met}$ (E. coli)	6.48	6.65	5.05	78
fMet-tRNA$_m^{Met}$ (E. coli)	2.62	2.82	2.12	81
fMet-tRNA$_f^{Met}$ (yeast)	4.86	4.86	3.60	74
fMet-tRNA$_m^{Met}$ (yeast)	3.98	3.80	3.10	78

The experiment was carried out as described in the text and in the legend to table 1. Each value represents an average from two experiments which were run under identical conditions.

[1] IF = crude initiation factors

[2] enzymatically formylated

E. coli could be observed. The results obtained with the two fMet-tRNA$_m^{Met}$ species from E. coli and yeast were not due to contaminations of the respective tRNA$_m^{Met}$ preparations with the corresponding tRNA$_f^{Met}$ species since neither of the two Met-tRNA$_m^{Met}$ preparations proved to be formylatable by E. coli transformylase. Fig. 2 illustrates the aminoacylation-formylation kinetics of the tRNA$_f^{Met}$ and tRNA$_m^{Met}$ (E. coli) used in this study. Whereas formylation of tRNA$_f^{Met}$ closely follows the aminoacylation curve, the formylmethionine bound to tRNA$_m^{Met}$ (E. coli) reaches a level slightly above background only after 40 minutes. Corresponding results were obtained for the two yeast tRNA^{Met} species (data not shown). Conceivably, chemical formylation of Met-tRNAs might not only lead to the formylation of the methionine moiety but could also introduce formyl groups into other positions of the tRNA-molecule. This in turn could alter the biological functions of a given tRNA. In order to test this possibility a sample of pure tRNA$_f^{Met}$ (E. coli) was treated with N-formyloxysuccinimide under the conditions used for chemical formylation (see "Experimental") and another sample was treated with formic acetic anhydride employing the conditions described for the acetylation of

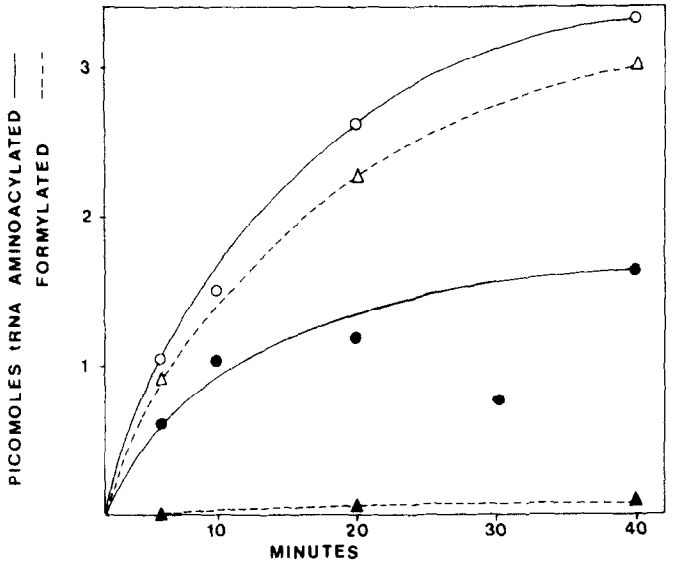

Fig. 2: Time course of aminoacylation and enzymatic formylation of Met-$tRNA_f^{Met}$ and Met-$tRNA_m^{Met}$ from E. coli. Each tube contained in a total volume of 0.1 ml 10µg of either of the two tRNAs, 10 µl of an aminoacyl synthethase preparation from E. coli, 10 µg of N^{10}-formyltetrahydrofolate, 1 µCi of [^{35}S]methionine, and other constituents as described previously (4, 5). Solid curves represent the rates of aminoacylation for $tRNA_f^{Met}$ (open circles) and $tRNA_m^{Met}$ (closed circles). The rates of formylation are represented by the dotted lines. Open triangles Met-$tRNA_f^{Met}$. Closed triangles Met-$tRNA_m^{Met}$.

Phe-tRNA with acetic anhydride (11). The two pretreated tRNAs and a corresponding sample of untreated material were then subjected to complete digestion with T_1 ribonuclease and the resulting fragments chromatographed on DEAE-Sephadex A25 columns (15). As shown in fig. 3, the elution profiles of fragmented control $tRNA_f^{Met}$ and of N-formyloxysuccinimide-treated material are virtually identical. Treatment with acetic-formic anhydride, on the other hand, appears to have inflicted a chemical alteration upon the tRNA documented by a somewhat changed elution profile of the corresponding T_1 digest. These results seem to indicate that the procedure of chemical formylation as used in this study does not give rise to the attachment of formyl groups to the tRNA molecule itself.

DISCUSSION: The data presented in this paper indicate that chain-internal methionyl-tRNAs can serve as substrates in the formation of initiation com-

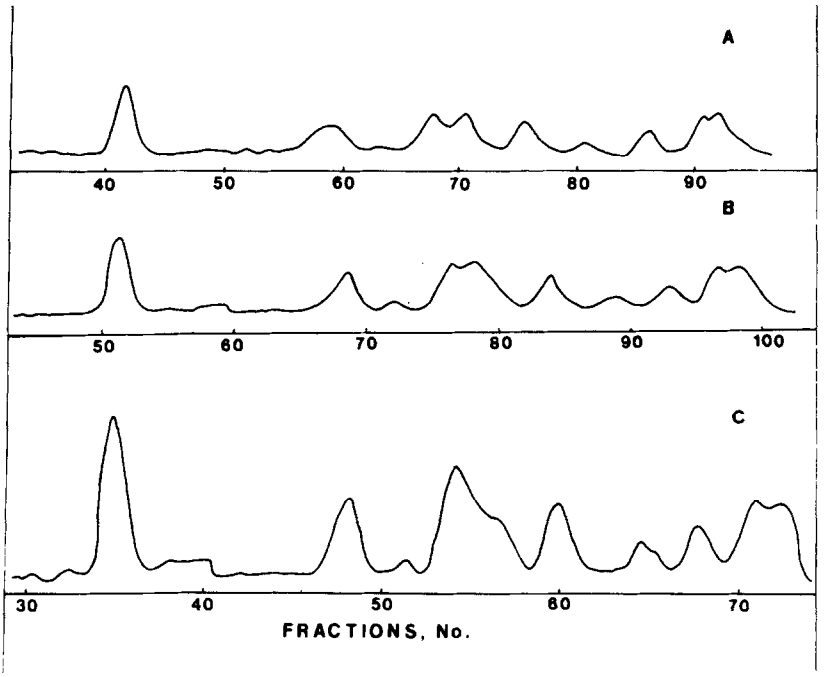

Fig. 3: Elution profiles of $tRNA_f^{Met}$ fragments prepared by controlled digestion with ribonuclease T_1. 7 A_{260} units of uncharged $tRNA_f^{Met}$ from E. coli (Boehringer) were kept under the conditions of chemical formylation (N-formylsuccinimide, 0° C for 10 min.). The material was then precipitated several times with alcohol and subjected to a complete digestion with T_1 ribonuclease (15). The resulting fragments were chromatographed on a DEAE-Sephadex A 25 column (0.5 x 148 cm) using a linear NaCl gradient of 0.15 - 0.7 M in 7 M urea and 0.02 Tris-HCl pH 7.5. Each chamber contained 100 ml of elution buffer (middle curve). Analogous experiments were carried out with 7 A_{260} units of $tRNA_f^{Met}$ which had been exposed to acetic-formic anhydride for 1 hour at 0° C (bottom curve) and with 5 A_{260} units of $tRNA_f^{Met}$ which had not been subjected to any pretreatment (upper curve).

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plexes in cell-free systems from E. coli, provided their methionine moieties are N-formylated. The ability of fMet-$tRNA_m^{Met}$ from E. coli and yeast to form functional initiation complexes with R 17 RNA and ribosomes from E. coli is distinctly smaller than the initiating capacity of fMet-$tRNA_f^{Met}$ from the same organisms. Moreover, initiation complexes formed with the chemically formylated Met-$tRNA_m^{Met}$ species from yeast and E. coli seem to be less stable during prolonged incubation periods than "physiological" initiation complexes including fMet-$tRNA_f^{Met}$ either from E. coli or yeast. These findings support the conclusion made by others on the basis of similar experiments (18) that

both the ribonucleic acid moiety and the presence of the formyl group represent important criteria for the recognition of a given Met-$tRNA^{Met}$ by bacterial initiation factors. However, the fact, that chemically formylated Met-$tRNA_m^{Met}$ from E. coli is clearly a better substrate for peptide chain initiation than unformylated Met-$tRNA_f^{Met}$ from the same organism emphasizes the dominating role of the formyl-methionine moiety for the recognition by bacterial initiation factors. We have recently shown that unformylated Met-$tRNA_f^{Met}$ from E. coli can serve as a donor of methionine into internal positions of growing peptide chains in a non-initiating cell-free system from E. coli (5). Corresponding results were presented by Ghosh and Ghosh (19). Taken together, these results and the findings reported in the present study indicate that the control providing the confinement of Met-$tRNA_f^{Met}$ to chain initiation and of Met-$tRNA_m^{Met}$ to chain elongation is exerted at several levels. Among these, N-formylation of the methionine moiety seems to be the most stringent one.

ACKNOWLEDGEMENT: The authors are indebted to G. Högenauer and G. Eder for help with the T_1 digestion of $tRNA_f^{Met}$ and to S. Johne, E. Neunteufel and E. Pichler for competent technical assistance.

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