PROTEIN CHAIN INITIATION BY METHIONYL-tRNA

Jonathan P. Leis and Elizabeth B. Keller

Biochemistry and Molecular Biology, Cornell University
Ithaca, New York 14850

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Summary. Wheat germ contains three chromatographically distinct methionine tRNA's, of which two species, one major and one minor, appear to function in protein chain initiation as demonstrated by the AUG-dependent reaction with puromycin on wheat germ ribosomes. Wheat germ extracts contain a very active transformylase which formylates the minor methionyl-tRNA. The major initiating methionyl-tRNA is not formylated or otherwise modified by wheat germ extracts. We postulate that protein chain initiation in the cytoplasm of wheat germ can be brought about by the major initiating methionyl-tRNA without prior formylation or other modification of the α amino group.

Since the discovery that N-formylmethionyl-tRNA (fMet-tRNA) (Marcker and Sanger, 1964) initiates protein synthesis in bacteria (Adams and Capecchi, 1966; Webster et al, 1966) a continuing search has been made for the tRNA or tRNA's serving the same function in eukaryotes. fMet-tRNA has not been found in the cytoplasm of eukaryotes but has been found in mitochondria (Smith and Marcker, 1968; Galper and Darnell, 1969) and chloroplasts (Schwartz et al, 1967; Burkard, et al, 1969). We sought a cytoplasmic initiating tRNA in a representative group of eukaryotes, yeast, wheat and beef. Taking fMet-tRNA as a model, we first searched for possible blocked aminoacyl-tRNA's. Separated tRNA's were charged by crude homologous cell extracts supplemented with N^{10}-formyltetrahydrofolate (N^{10}-fTHF) and acetyl-CoA. Charging was then reversed by adding excess AMP and pyrophosphate. Blocking of the aminoacyl-tRNA prevents this reversal. Using this assay, it was discovered that in wheat germ there is a minor methionine tRNA ($tRNA^{Met}$) which can be formylated by a wheat germ extract (Leis and Keller, 1970). This tRNA is probably derived from plastids and/or mitochondria, since it is only 4% of the total $tRNA^{Met}$. Its cellular localization is being investigated. In all other cases, the assay gave negative results. This led us to consider initiation by an unblocked aminoacyl-tRNA. From evolutionary considerations, unblocked methionyl-tRNA

(Met-tRNA) was a likely possibility. This paper presents evidence for such a cytoplasmic initiating Met-tRNA in wheat germ.

Results. Wheat germ contains three species of $tRNA^{Met}$, two major and one minor, which can be separated on benzoylated DEAE-cellulose (BD-cellulose) columns (Gillam et al, 1967). When bulk tRNA is fractionated in the presence of EDTA at pH 4.5 (Fig. 1) the two major species are well resolved. The minor species, $tRNA_3$, elutes at 0.76 M NaCl and is not completely resolved from $tRNA_2$. It can be separated from the two major tRNA's by chromatography on a BD-cellulose column in the presence of 10 mM $MgCl_2$.

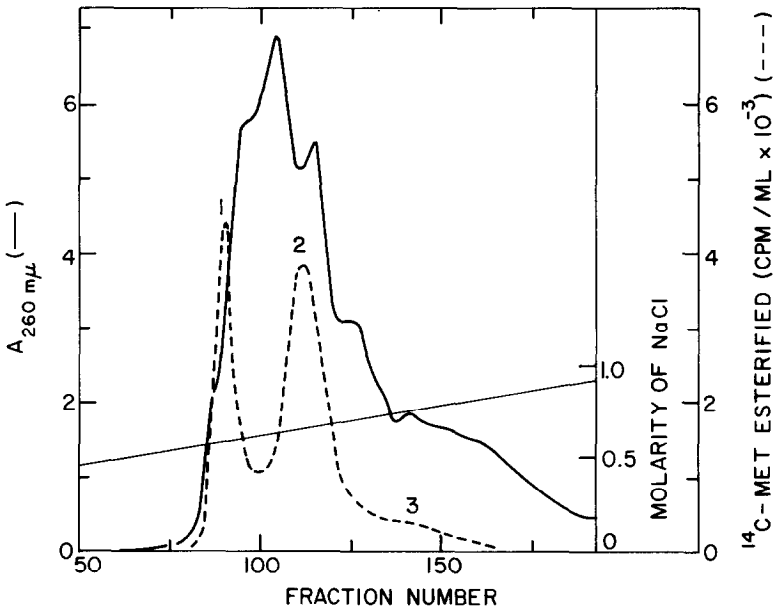

Fig. 1. Fractionation of wheat germ tRNA on BD-cellulose in the presence of EDTA at pH 4.5. 125 mg of bulk tRNA prepared from commercially processed wheat germ (Dudock et al, 1969) was adsorbed to a BD-cellulose column (1.0 x 90 cm) equilibrated with 0.3 M NaCl, 1 mM EDTA, 10 mM NaOAc (pH 4.5) and eluted with a 1.4 l linear gradient from 0.3 M to 1.0 M NaCl containing 1 mM EDTA, 10 mM NaOAc (pH 4.5). The collection tubes contained a concentrated solution of Tris·HCl (pH 7.5) and $Mg(OAc)_2$ to bring the pH of the fractions to 7 and the (Mg^{++}) to 10 mM. Fractions of 7 ml were collected at a flow rate of 1 ml/min and were assayed for acceptor activity (Loehr and Keller, 1968) using ^{14}C-methionine (10 mc/mmole) and a purified wheat germ soluble enzyme (Yoshikami, 1970). Counting was in a thin-window gas flow counter (efficiency 31%).

$Met\text{-}tRNA_3$ is formylated by a transformylase from wheat germ extracts. The optimum conditions for this reaction are given in Fig. 2. Transformylation

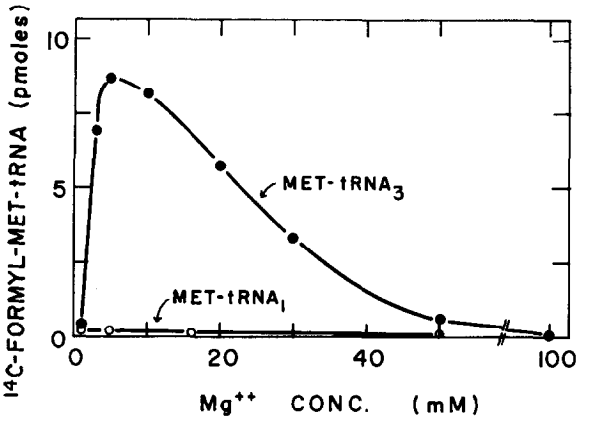

Fig. 2. Transformylation of methionyl-tRNA with wheat germ transformylase. The assay contained in a volume of 0.10 ml; 50 mM Tris·HCl (pH 7.5), 20 mM KCl, 6 mM dithiothreitol, $MgCl_2$ and Met-tRNA as indicated, 11 uM N^5,N^{10}-^{14}C-methenyltetrahydrofolate (23.6 mc/mmole) (Rabinowitz and Pricer, 1962) held at pH 8 under argon for 90 seconds to form N^{10}-^{14}C-fTHF before addition (Huennekens, 1963), and 0.2 mg/ml wheat germ soluble enzyme fraction (see Fig. 1). (-●-) ^{12}C-Met-tRNA$_3$ (10 pmoles) isolated by phenol treatment and Sephadex G-25 chromatography from a preparative charging incubation. (-○-) ^{12}C-Met-tRNA$_1$ (60 pmoles). Incubation was for 5 min at 37°. The ^{14}C-formylmethionyl-tRNA was precipitated, collected on a glass fiber membrane and counted as in Fig. 1.

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of Met-tRNA's 1 and 2 was attempted under a wide variety of conditions, always with negative results. For example, Met-tRNA$_1$ is not formylated at Mg^{++} concentrations from 1 to 50 mM (Fig. 2) or K^+ concentrations up to 0.1 M. Met-tRNA$_1$ is also not formylated by E. coli transformylase.

The reaction of ^{14}C-Met-tRNA's with puromycin was used to distinguish binding at the initiating (peptidyl) and aminoacyl-tRNA sites (Bretscher and Marcker, 1966) on wheat germ ribosomes. Ethyl acetate extraction at pH 8 made possible the detection of both methionyl- and formylmethionyl-puromycin (Leder and Bursztyn, 1966). The products were identified by electrophoresis at pH 3.5. Met-tRNA$_1$, bound to the ribosomes with AUG in a preincubation, rapidly forms methionyl-puromycin upon the addition of puromycin (Fig. 3A). Met-tRNA$_2$, however, does not give a significant initial synthesis of methionyl-puromycin (Fig. 3A). The AUG-dependent binding under these conditions was determined in a separate incubation (Nirenberg and Leder, 1964) to be 1.3 pmoles

bound per 3 A_{260} units of wheat germ ribosomes. In spite of this appreciable AUG-dependent binding, there was no initial puromycin reaction with Met-$tRNA_2$. We conclude, therefore, that Met-$tRNA_1$ is an initiating tRNA and Met-$tRNA_2$ is not. In an incorporation experiment with random poly (A,U,G), Met-$tRNA_2$ was

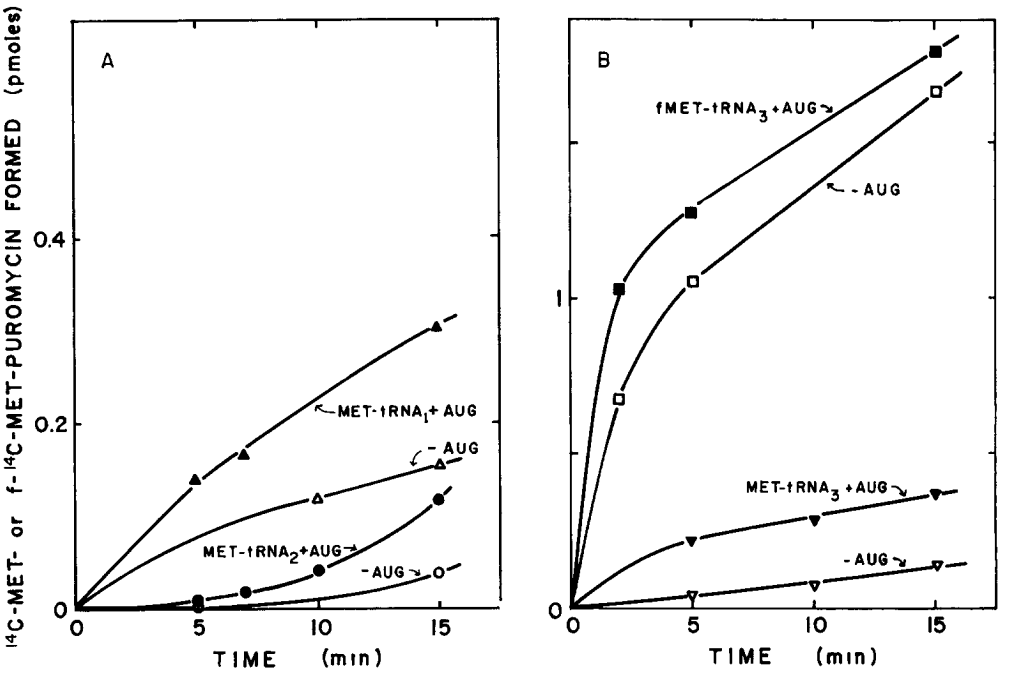

Fig. 3. Formation of Met- and fMet-puromycin in the presence of wheat germ ribosomes. The assay was in two steps. 1) AUG-dependent binding of Met- or fMet-tRNA to ribosomes. The incubation mixture contained in 0.045 ml, 55 mM Tris·HCl (pH 7.5), 55 mM KCl, 11 mM $MgCl_2$, 2.2 mM dithiothreitol, 0.66 mM GTP, 0.1 A_{260} units AUG where indicated, 3 A_{260} units wheat germ ribosomes, and 20-30 pmoles charged tRNA prepared as in Fig. 2 but with ^{14}C-methionine (200 mc/mmole) (fTHF added where indicated). The ribosomes were prepared from commercial wheat germ and were unwashed (Yoshikami, 1970). Incubation was for 4 min at 37°. 2) Puromycin reaction. Puromycin was added to give a final concentration of 1 mM. The final volume was increased to 0.05ml. Reincubation was at 37°. The reaction was stopped with the addition of 1 ml 0.1 M Na_2HPO_4 (pH 8.1) and 1.5 ml ethyl acetate (saturated with Na_2HPO_4 buffer). The phases were mixed on a Vortex mixer 10 times, 5 seconds each, then separated by centrifugation. A 1 ml aliquot of the ethyl acetate layer was removed for counting with 10 ml Bray's scintillation fluid (Bray, 1960) in a Packard scintillation counter (efficiency, 75%). The values plotted have been corrected for the blanks run without puromycin. A. Comparison of Met-tRNA's 1 and 2. (-▲-) ^{14}C-Met-$tRNA_1$ plus AUG, (-△-) same minus AUG; (-●-) ^{14}C-Met-$tRNA_2$ plus AUG, (-○-) same minus AUG; B. Effect of formylation of Met-$tRNA_3$. (-▼-) ^{14}C-Met-$tRNA_3$ plus AUG, (-▽-) same minus AUG; (-■-) formyl-^{14}C-Met-$tRNA_3$ (prepared as above with N^5,N^{10}-^{12}C-methenyltetrahydrofolate (Huennekens, 1963) added as in Fig. 3) plus AUG, (-□-) same minus AUG.

found to insert methionine into the growing polypeptide chain. Thus it is a $tRNA_m^{Met}$.

Brown and Smith (1970), using a mouse ascites tumor system, found that formylation of Met-$tRNA_f$* destroys its initiating ability. They attribute this to some property of the 80 S cytoplasmic ribosomes used. The wheat germ ribosomes used in the present experiments were also 80 S (1% 70 S), yet formylation of Met-$tRNA_3$ enhanced the rate of its reaction with puromycin (Fig. 3B). If a small amount of fMet-$tRNA_3$ were present in the cytoplasm, some cytoplasmic protein could be synthesized with N-terminal fMet- because of this enhanced rate.

Discussion. In the fractionation of the $tRNA^{Met}$'s of eukaryotes (Caskey et al, 1967; Takeishi et al, 1968; RajBhandary and Ghosh, 1969; Smith and Marcker, 1970) only two species have been detected, a Met-$tRNA_m$ and a Met-$tRNA_f$* (formylatable by E. coli transformylase). Smith and Marcker have proposed that Met-$tRNA_f$* is cytoplasmic. These fractionations have failed so far to show a distinct mitochondrial fMet-$tRNA_f$, though total cell tRNA was used in each case.

In the present study on wheat germ $tRNA^{Met}$'s, three distinct species have been separated, two initiating and one $tRNA_m^{Met}$. The minor Met-$tRNA_3$ is formylated by the transformylase in wheat germ extracts and is probably chain initiating in the organelles (cf. Burkard et al, 1969). The major Met-$tRNA_1$ is a chain initiating tRNA as indicated by the reaction with puromycin. It is probably cytoplasmic in origin as it makes up about 2% of the total tRNA of the cell. It cannot be formylated by the transformylase present in wheat germ extracts. We propose, therefore, that it functions for chain initiation in the cytoplasm without prior formylation. We suggest that this type of initiating tRNA be called $tRNA_i^{Met}$.

Galper and Darnell (1969) reported that inhibition of formylation by aminopterin did not affect the growth of HeLa cells. Their results are consistent with the above postulate. Brown and Smith (1970), using the mouse

ascites tumor system, have found that initiation by Met-$tRNA_f$* is not dependent upon formylation at low Mg^{++} concentrations. Further evidence for the proposed mechanism was recently obtained by Wilson and Dintzis (1970) who have detected unblocked methionine as the N-terminal amino acid of growing globin chains on reticulocyte ribosomes. Similar results have been observed in an in vitro wheat germ system with TMV-RNA as messenger by D. Weeks, T. Schoolt, and A. Marcus (personal communication). Together with the results on wheat germ reported here, this evidence from a variety of systems suggests that an unblocked Met-tRNA may function for protein chain initiation in the cytoplasm of eukaryotic organisms.

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