STUDIES ON THE INITIATION OF PROTEIN SYNTHESIS IN ANIMAL TISSUES

S.S. Kerwar, Carlos Spears, and Herbert Weissbach

Roche Institute of Molecular Biology
Nutley, New Jersey 07110

Received August 21, 1970

SUMMARY: Two methionine accepting tRNAs have been separated from rabbit liver tRNA by column chromatography on BD-cellulose. One of the species (Met-$tRNA_F^{Met}$) can be formylated using an Escherichia coli transformylase preparation, and maximal binding of this species to brain ribosomes is achieved in the presence of AUG, GTP and a 0.5 M NH_4Cl extract of the ribosomes. Transferase I cannot replace the NH_4Cl extract in the binding assay. The other tRNA species (Met-$tRNA^{Met}$) requires GTP and transferase I for binding to ribosomes.

Although the role of fMet-$tRNA_F^{Met}$[1] in the initiation of protein synthesis in bacteria has been extensively studied (1), the role of this tRNA species in the initiation process in eukaryotic cells has only recently received much attention. Caskey et al. (2) had previously shown that guinea pig liver contains two species of methionine accepting tRNAs, one of which could be formylated with $N^{5,10}$-methenyl-H_4-folate in the presence of an E. coli transformylase preparation. Smith et al. (3) and Brown et al. (4) have recently provided convincing evidence indicating that the Met-$tRNA_F^{Met}$, the tRNA species which can be formylated by the E. coli transformylase acts as an initiator tRNA in eukaryotic cells. Using an amino acid polymerization assay, these authors have shown that AUG is the initiator codon, but unlike the results in the bacterial system (1), the incorporation of methionine of Met-$tRNA_F^{Met}$ occurs without prior formylation.

The present communication describes studies on the binding to ribosomes of the two animal Met-tRNA species. The results show that specific factors present in a salt extract of the ribosomes are required for the binding of Met-$tRNA_F^{Met}$ to the ribosomes, while transferase I is required for the binding of Met-$tRNA^{Met}$.

1 The following abbreviations have been used: $tRNA_F^{Met}$, methionine accepting species that can be formylated; $tRNA^{Met}$, methionine accepting species that cannot be formylated; H_4-folate, tetrahydrofolate; BD-cellulose, benzoylated diethylaminoethyl cellulose.

METHODS: Deacylated tRNA of rabbit liver was obtained from General Biochemicals, Chagrin Falls, Ohio. ^{3}H-methionine (7 c/mmole, 4500 cpm/μμmole) was purchased from Amersham Searle, Chicago, Illinois. BD-cellulose (50-100 mesh) was a product of Schwarz Biochemicals, New York. AUG and poly AGU (3:1:1) were obtained from Miles Laboratories.

Acylation of rabbit liver tRNA with methionine was achieved by using a 35-65% ammonium sulfate fraction of calf brain which contained the amino acid activating enzymes (2). Ribosomes were prepared from predominantly grey matter of calf brain by the method of Goodwin et al. (5). The ribosome pellet was washed with 0.5 M NH_4Cl containing 1 mM Mg^{++} and 100 mM Tris-Cl buffer, pH 7.4. The ammonium chloride extract was saved. The ribosomes were then resuspended in the above buffer and pelleted through a 1 M sucrose solution (RNase free) at 280,000 x g for 3 hrs. The ribosomal pellet was suspended in sucrose buffer as described by Goodwin et al. (5).

Separation of Met-$tRNA_F^{Met}$ and Met-$tRNA^{Met}$ was achieved by column chromatography on BD-cellulose. Approximately 630 (A_{260}) O.D. units of rabbit liver tRNA acylated with ^{3}H-methionine (1.2% total Met acceptance) were layered on a BD-cellulose column (2.5 x 30 cm) previously washed with 0.02 M sodium acetate buffer, pH 5.0, containing 0.45 M NaCl, 10 mM Mg^{++} and 1 mM mercaptoethanol. The column was developed at 4° using a salt gradient with 1500 ml of 0.45 NaCl buffer in the mixing chamber and an equal volume of 0.9 M NaCl in the reservoir. Twelve ml fractions were collected (flow rate of approximately 1 ml/min). Fractions were scanned for TCA precipitable radioactivity and for absorbancy at 260 mμ (Fig. 1). Met-$tRNA_F^{Met}$, which can be formylated using an E. coli transformylase preparation and $N^{5,10}$-methenyl-H_4-folate, elutes between fractions 65-100, whereas Met-$tRNA^{Met}$, which is not a sbustrate for the transformylase enzyme elutes between fractions 140-210. The extent of formylation of Met-$tRNA_F^{Met}$ was approximately 30% of the input concentration (7 μμmoles) of the tRNA species. Similar values were obtained by Caskey et al. (2) using guinea pig liver Met-$tRNA_F^{Met}$. Fractions from each peak were pooled, lyophilized, and desalted on a Sephadex G-25 column using 1 x 10^{-3} M

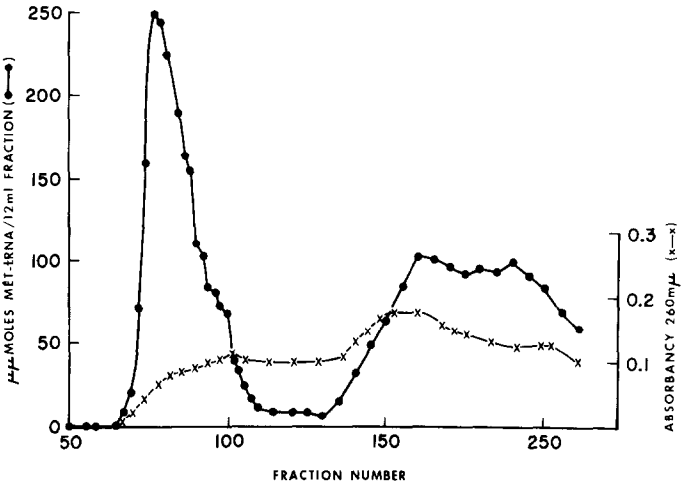

Fig. 1. Separation of Met-tRNA$_F^{Met}$ and Met-tRNA^{Met} by BD-cellulose column chromatography.

cacodylate buffer, pH 5.5, containing 1 mM Mg^{++}. Desalted fractions were analyzed for TCA precipitable radioactivity and then were lyophilized to dryness and dissolved in a minimum amount of water.

Formylation assays of Met-tRNA$_F^{Met}$ and Met-tRNA^{Met} were performed using a purified E. coli transformylase fraction prepared by the method of Dickerman et al. (6), and kindly supplied by Dr. Dickerman. Binding of Met-tRNA$_F^{Met}$ and Met-tRNA^{Met} to ribosomes was measured by a modification of the method of Nirenberg and Leder (7). The ribosomal binding assay in a total volume of 75 μl contained: 5-8 mM magnesium acetate, 50 mM Tris-acetate buffer, pH 7.4; 50 mM potassium acetate, ribosomes (A_{260} = 1.2), 1.3 mM GTP, either the 0.5 M NH_4Cl ribosomal extract or transferase I, AUG (A_{260} = 1.3) or poly AGU (3:1:1, 5 μg) and 5 μμmoles of either Met-tRNA$_F^{Met}$ or Met-tRNA^{Met} (specific activity 4500 cpm/μμmole). Unless stated otherwise incubations were at 37° for 4 min. The reactions were stopped by the addition of 2.5 ml of cold buffer (50 mM Tris-Cl buffer, pH 7.4; 160 mM NH_4Cl and 12 mM $MgCl_2$) and the solution was filtered through a 0.45 μ nitrocellulose filter (Millipore Corp.). The filter was washed three times with 2.5 ml of the above buffer and dissolved in a napthalene-dioxane solution (8) and assayed for radioactivity.

Purified transferase I was a gift from Dr. B.S. Baliga of Massachusetts Institute of Technology and was prepared from rat liver by the method of Gasior and Moldave (9). Protein concentrations were determined by the method of Warburg and Christian (10).

RESULTS: Binding of Met-tRNA$_F^{Met}$ and Met-tRNA^{Met} to Ribosomes. The requirements for the binding of Met-tRNA$_F^{Met}$ to ribosomes are shown in Table I.

TABLE I

Requirements for Binding of Met-tRNA$_F^{Met}$ to Ribosomes

System	Met-tRNA$_F^{Met}$
	μμmoles bound
Complete	0.15
- AUG	0.00
- GTP	0.13
- Ribosomal extract	0.05
- Ribosomal extract + transferase I	0.05

Details of the assay are described in the text. Where indicated 50 μg of ribosomal extract protein and 10 μg of transferase I protein were added. Radioactivity retained on the filter in a system in which ribosomes were omitted has been subtracted.

Binding of this species is dependent on AUG and stimulated between 3 to 5 fold by the addition of the 0.5 M NH_4Cl extract of the ribosomes. Transferase I could not replace the ribosomal extract. A slight stimulatory effect of GTP on the binding (ranging between 15-35%) was routinely observed. The kinetics of binding of Met-tRNA$_F^{Met}$ are shown in Fig. 2. Binding increases with time and is dependent on the ribosomal extract throughout the time of incubation. In contrast, the binding of Met-tRNA^{Met} to ribosomes was completely dependent on GTP and transferase I; the latter could not be replaced by the ribosomal extract (Table II).

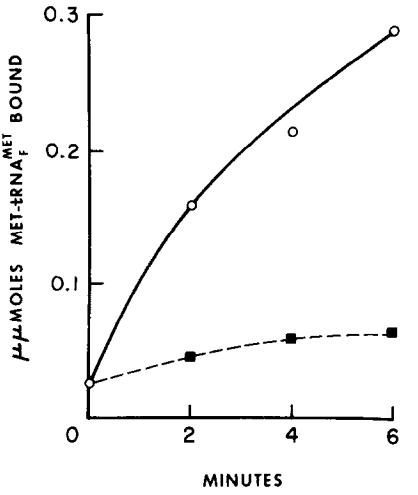

Fig. 2. Kinetics of binding of Met-tRNA$_F^{Met}$ to ribosomes. (■ ---■) Binding in the absence of ribosomal extract; (○ —— ○) binding in the presence of 50 μg of ribosomal extract protein. Other details of the assay are described in the text.

TABLE II

Cofactor Requirements for Transferase I Dependent Binding of Met-tRNA^{Met} to Ribosomes Using AUG or poly AGU

System	AUG	Poly AGU
	μμmoles bound	
Complete	0.12	0.45
- AUG or poly AGU	0.11	0.13
- GTP	0.00	0.00
- Transferase I	0.00	0.00
- Transferase I + ribosomal extract	0.03	0.01

The amounts of transferase I and ribosomal extract are as described in Table I. Other details are described in the text.

The triplet AUG did not stimulate the binding of Met-tRNA^{Met} to ribosomes although when AUG was replaced by poly AGU, a 3-fold stimulation in binding could be demonstrated (Table II).

DISCUSSION: In a recent communication, Smith, et al. (3) have provided evidence that Met-tRNA$_F^{Met}$ of eukaryotic cells incorporates methionine in the N-terminal position of newly synthesized protein in response to poly AUG or poly UG, whereas Met-tRNA^{Met} is responsible for inserting methionine residues into the internal positions of the protein. These authors have proposed that Met-tRNA$_F^{Met}$ of eukaryotic cells can serve as an initiator of protein synthesis in an analogous fashion to the role of fMet-tRNA$_F^{Met}$ in bacterial systems. If true, one would expect eukaryotic cells to also have a specific mechanism for the binding of Met-tRNA$_F^{Met}$ to ribosomes. The results presented here provide additional evidence for the initiator role of Met-tRNA$_F^{Met}$ in animal cells. The binding to brain ribosomes of Met-tRNA$_F^{Met}$ is dependent on AUG and stimulated between 3 to 5 fold by the addition of a 0.5 M NH_4Cl ribosomal extract. The ribosomal extract cannot be replaced by transferase I. Miller and Schweet (11) first showed that an extract of ribosomes was involved in the initiation of protein synthesis in rabbit reticulocytes, and their findings have recently been extended by Prichard et al. (12). However, an interaction of the ribosomal factors with a specific tRNA has not been demonstrated previously. It is probable from the present results that the factors present in the salt extract of ribosomes promote the formation of an initiation complex consisting of ribosomes, messenger, and initiator tRNA. A similar system, dependent on GTP, has been described in bacterial systems (13). Although the results in Table I show only a small dependency on GTP for the binding of Met-tRNA$_F^{Met}$ to ribosomes, this may be due to the presence of nucleotides in the crude ribosome extract.

Binding of Met-tRNA^{Met} to ribosomes is completely dependent on transferase I and GTP. A transferase I and GTP dependent binding of aminoacyl-tRNA to ribosomes has been studied in detail in systems from rabbit reticulocytes and liver (14,15). One troublesome aspect of the present studies is the lack of stimulation of the binding of Met-tRNA^{Met} by the triplet AUG (Table II), although the reaction is stimulated by the copolymer AGU and the AUG triplet does stimulate the binding of Met-tRNA$_F^{Met}$ to ribosomes (Table I). This difference in the

response of the two Met-tRNA species to AUG has not yet been explained.

The present results show that transferase I, like Tu in the bacterial system, does not interact with Met-$tRNA_F^{Met}$, thus providing a mechanism to prevent the incorporation of methionine from Met-$tRNA_F^{Met}$ into the internal positions of the protein. In addition, the specificity of the ribosome extract for Met-$tRNA_F^{Met}$ should direct the incorporation of methionine from Met-$tRNA_F^{Met}$ into the N-terminal position of the synthesized protein.

REFERENCES

1. Lengyel, P., and Söll, D. Bacteriol. Rev. 33, 264 (1969).
2. Caskey, C.T., Redfield, B., and Weissbach, H. Arch. Biochem. Biophys. 120, 119 (1967).
3. Smith, A.E., Marcker, K.A. Nature 226, 607 (1970).
4. Brown, J.C., and Smith, A.E. Nature 226, 610 (1970).
5. Goodwin, F., Shafritz, D., and Weissbach, H. Arch. Biochem. Biophys. 130, 183 (1969).
6. Dickerman, H., Steers, E., Redfield, B.G., and Weissbach, H. J. Biol. Chem. 242, 1522 (1967).
7. Nirenberg, M., and Leder, P. Science 145, 1399 (1964).
8. Bray, A.G. Anal. Biochem. 1, 279 (1960).
9. Gasior, E., and Moldave, K. J. Biol. Chem. 240, 3346 (1965).
10. Warburg, O., and Christian, W. Biochem. Z. 310, 384 (1941).
11. Miller, R.L., and Schweet, R. Arch. Biochem. Biophys. 125, 632 (1968).
12. Prichard, P.M., Gilbert, J.M., Shafritz, D.A., and Anderson, W.F. Nature 226, 511 (1970).
13. Stanley, W.M., Jr., Salas, M., Wahba, A.J., and Ochoa, S. Proc. Nat. Acad. Sci. 56, 290 (1966).
14. Moldave, K., Ibuki, F., Rao, P., Schneir, M., Skogerson, L., and Sutter, R.P. In "Regulatory Mechanisms for Protein Synthesis in Mammalian Cells" A. San Pietro, M.R. Lamborg, and F.J. Kenney (Eds.), Academic Press, New York, pp. 191-230 (1968).
15. Hardesty, B., Culp, W., and McKeehan, W. Cold Spring Harbor Symp. Quant. Biol. 34, 331 (1970).