DISCRIMINATION BY RAT LIVER AMINOACYLTRANSFERASE I AGAINST Met-tRNA$_{F*}$

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SUMMARY

The two species of methionine-accepting tRNA from rat liver have been separated and their enzymatic binding to ribosomes studied. At a Mg^{++} concentration of 0.006 M, rat liver aminoacyltransferase I promotes the binding of the non-formylatable species of Met-tRNA to 80 S ribosomes in the presence of poly AUG. The formylatable species, however, is not bound to ribosomes by this purified transfer factor.

Two species of mammalian methionine-accepting tRNA have been separated (1,2). One species (Met-tRNA$_{F*}$) can be formylated by E. coli formylase while the other (Met-tRNA$_{M*}$) cannot be. Since methionine attached to $tRNA^{Met}_{M*}$ was incorporated into internal positions of polypeptides while methionine attached to $tRNA^{Met}_{F*}$ was incorporated into N-terminal positions, Smith, Marcker, and Brown (2,3) suggested that Met-tRNA$_{F*}$ is an initiator aminoacyl-tRNA for mammalian protein synthesis. In fact methionine has been identified as the amino-terminal residue of short nascent chains in vivo and in vitro (4-6). Since no formylase and no fMet-tRNA have been detected in the cytoplasm of mammalian cells, it is assumed that Met-tRNA$_{F*}$, without being formylated, can serve as an initiator (2).

In this report we show that rat liver aminoacyltransferase I, the soluble factor which binds aminoacyl-tRNA to ribosomes during chain elongation, stimulates the binding of Met-tRNA$_{M*}$ to ribosomes in the presence of poly AUG, but fails to recognize Met-tRNA$_{F*}$.

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Discrimination by the transfer factor against Met-tRNA$_{F*}$ explains the failure of this aminoacyl-tRNA to donate methionine to internal positions in a chain, although the amino group is not protected. Similar results were reported for a bacterial system (7).

MATERIALS AND METHODS

Separation of two species of tRNA^{Met}: Separation procedures are similar to those of Smith and Marcker (2). Benzoylated DEAE-cellulose (Schwarz, mesh: 50-100) was washed with 10% ethanol, 1.5 M NaCl. A 10 x 1 cm column was packed and equilibrated with 0.45 M NaCl, 0.005 M $MgCl_2$, 0.001 M 2-mercaptoethanol. Rat liver tRNA (10 mg; General Biochemicals) was dissolved in 1 ml of equilibrating solution and applied to the column. tRNA was eluted at 5°C with a linear NaCl gradient from 0.45 M to 0.9 M, containing 0.005 M $MgCl_2$, 0.001 M 2-mercaptoethanol. Two ml fractions were collected.

Assay of methionine accepting activity: Conditions for assaying methionine accepting activity followed Nishimura and Weinstein (8). A 0.1 ml reaction mixture contained 0.1 M Tris-HCl, pH 7.2, 0.01 M magnesium acetate, 0.01 M KCl, 0.002 M ATP, 0.1 µCi ^{14}C-methionine (200 mCi/mmole; New England Nuclear), varying amounts of tRNA, and 0.81 mg of aminoacyl-tRNA synthetase (8). Incubation was at 37°C for 10 minutes. ^{14}C-Met-tRNA was precipitated with 5 ml of 5% cold trichloracetic acid (TCA), collected on a glass fiber filter (Whatman) and washed twice with 5 ml cold TCA. The precipitate was digested with 0.2 ml of NCS solubilizer (Amersham/Searle) and counted with 5 ml of a Liquifluor (New England Nuclear)-toluene scintillation cocktail in a Nuclear Chicago Mark I Scintillation Counter.

Formylation of Met-tRNA: Conditions for formylation of Met-tRNA were similar to the assay for methionine accepting activity of tRNA except that the reaction mixture contained 2.5 x 10^5 cpm of $N^{5,10}$-^{14}C-methenyltetrahydrofolate, purified E. coli formylase (both gifts

of Dr. Herbert Weissbach), 0.5 μmole of cold methionine, and tRNA as indicated in individual experiments. The reaction mixture was incubated for 30 minutes at 30°C. 5 ml of 5% cold TCA were then added. The precipitate was immediately collected on a filter (Millipore Corporation, HAWP, 0.45 μ) and washed twice with 5 ml cold 5% TCA (9). The filters were counted in Bray's solution (10).

Preparation of poly AUG: Poly AUG (1:1:1) was synthesized following published procedures (11,12) with polynucleotide phosphorylase (P-L Biochemicals). We thank Dr. Frank B. Howard for help in the synthesis.

Binding assay of Met-tRNA to ribosomes: Purified rat liver transferase I was used. Details for the preparation, which had only two bands in analytical disc gel electrophoresis, will be described eleswhere. Rat liver ribosomes, free of endogenous mRNA and elongation factors, were prepared by the method of Martin and Wool (13). The two species of $tRNA^{Met}$ were charged separately with ^{3}H or ^{14}C-methionine and Met-tRNA was isolated by phenol extraction and ethanol precipitation. The binding of Met-tRNA to ribosomes was done following Nirenberg and Leder (14). A 0.5 ml reaction mixture contained 0.006 M $MgCl_2$, 0.04 M NH_4Cl, 0.01 M dithiothreitol, 0.0003 M GTP, 0.05 M Tris-HCl, pH 7.2, varying amounts of transferase I, 170 μg ribosomes, 3.5 A_{260} units of poly AUG, and ^{3}H or ^{14}C-Met-tRNA containing 9 to 10 pmoles of methionine. The reaction mixture was incubated at 37°C for 10 minutes and 5 ml of cold 0.006 M $MgCl_2$, 0.08 M NH_4Cl, 0.05 M Tris-HCl, pH 7.2, were added. The ribosomes were collected on a millipore filter and counted in Bray's solution.

RESULTS

Separation of two species of Met-tRNA: Two species of rat liver $tRNA^{Met}$ were separated by benzoylated DEAE (BD) cellulose column chromatography (2). Met-tRNA from the two major peaks were separately pooled and assayed for formylation (Table 1). As shown previously

Table 1. Formylation of methionine-tRNA.

$tRNA^{Met}$	Formylase	CPM
Peak 1	+	2820
Peak 1	-	366
Peak 2	+	248
Peak 2	-	247

$tRNA^{Met}$ from the two peaks of the BD-cellulose column was pooled separately and dialyzed against distilled water. 0.3 ml from each pooled peak was used for the assay of formylation.

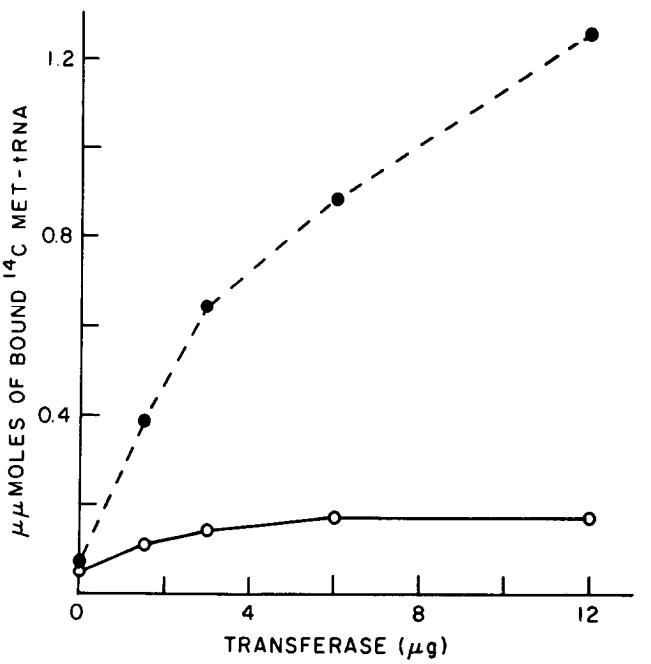

Figure 1. Transferase I-dependent binding of two species of Met-tRNA to ribosomes. Experimental conditions are the same as described in Methods. 10 pmoles of ^{14}C-Met-$tRNA_{M*}$ and ^{14}C-Met-$tRNA_{F*}$ were assayed with varying amounts of transferase I.

●---●---● met-$tRNA_{M*}$;

0—0—0 Met-$tRNA_{F*}$.

by Smith and Marcker (2), the first peak which elutes ($tRNA_{F*}^{Met}$) can be formylated, but the second peak ($tRNA_{M*}^{Met}$) cannot.

Binding of two species of Met-tRNA to 80 S ribosomes: To determine if the binding of both species of Met-tRNA to 80 S rat liver ribosomes is stimulated by rat liver transferase I, tRNA from the two peaks was separately charged with ^{14}C-methionine. The binding of each species to ribosomes at 0.006 M Mg^{++} was studied at various levels of transferase I (Fig. 1). The binding of ^{14}C-Met-$tRNA_{M*}$ was increased more than ten-fold by added transferase I, while ^{14}C-Met-$tRNA_{F*}$ showed a very low binding of unknown significance. The transferase I-dependent binding of Met-$tRNA_{M*}$ to ribosomes requires poly AUG and GTP (Table 2).

In Table 3, the binding of unfractionated Met-tRNA and of Met-$tRNA_{M*}$ is compared. In the presence of equal amounts of Met-tRNA, the amount of Met-$tRNA_{M*}$ bound to ribosomes is more than twice that of unfractionated Met-tRNA. If the efficiency of charging methionine for both species of

Table 2. Requirements for the binding of Met-$tRNA_{M*}$ to ribosomes.

Omissions	pmoles Met-$tRNA_{M*}$ bound
None	1.65
Poly AUG	0.41
Ribosomes	0.00
Transferase I	0.05
GTP	1.02

The complete system for the binding is described in Methods. The amount of ribosomes was 450 µg, and of ^{3}H-Met-$tRNA_{M*}$ was 8.6 pmoles.

Table 3. Binding of Met-tRNA$_{M*}$ and unfractionated Met-tRNA to ribosomes.

Met-tRNA	Bound Met-tRNA (pmoles)
^{14}C-Met-tRNA$_{M*}$	0.955
Unfractionated ^{14}C-Met-tRNA	0.411

Nine pmoles of ^{14}C-Met-tRNA$_{M*}$ and of unfractionated ^{14}C-Met-tRNA were assayed. Experimental conditions are the same as described in Methods.

of tRNA^{Met} in the mixture in the mixture is the same, then one might estimate that tRNA$^{Met}_{M*}$ comprises approximately 40% of total tRNA^{Met}.

DISCUSSION

These experiments confirm previous work that there are two species of mammalian tRNA^{Met}, of which only one can be formylated (1,2). Approximately 40% of the total tRNA^{Met} from rat liver is tRNA$^{Met}_{M*}$. Further, we have shown that rat liver transferase I does not recognize Met-tRNA$_{F*}$ for binding to ribosomes while it does recognize Met-tRNA$_{M*}$. As a corollary this explains why Met-tRNA$_{F*}$ cannot donate methionine to the internal positions of polypeptides whereas Met-tRNA$_{M*}$ can (2). These experiments also have prompted us to look for one or more protein factors in rat liver which will recognize Met-tRNA$^{Met}_{F*}$ for binding to ribosomes. Preliminary data indicate that such a factor(s) is present in the 1 M KCl ribosomal wash. Shafritz and Anderson (15) have recently shown that transferase I from rabbit reticulocytes binds Met-tRNA$_{M*}$ to ribosomes and that two of the reticulocyte initiation factors together bind the formylatable species.

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