Initiation of Protein Synthesis in Yeast: Binding of Met-tRNA$_i$

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Conditions for the binding of Met-tRNA$_i$ to 40 s ribosomal subunits and to proteins isolated out of the yeast ribosomal KCl wash were investigated. Sucrose density gradient experiments revealed that binding of Met-tRNA$_i$ to 40 s ribosomal subunits was catalyzed in a AUG and GTP dependent reaction. Binding of Met-tRNA$_i$ to proteins of the ribosomal KCl wash as assayed by the Millipore filter technique was found to be independent of AUG, GTP and 40 s ribosomal subunits. Additions of GTP yielded only slight stimulation, whereas Mg^{2+} caused dissociation of complexes. It was concluded that these reactions were most likely catalyzed by initiation factor eIF-2 although stimulation by GTP did not occur.

Introduction

Since Levin *et al.* [1] found an initiation factor in L-cells which bound GTP and Met-tRNA$_i$, subsequent investigations of the initiation of eukaryotic protein synthesis, for the most part in mammalian cells, *Artemia salina* and wheat germ led to the generally accepted concept that ternary complex (eIF-2 · GTP · Met-tRNA$_i$) formation is necessary before binding of Met-tRNA$_i$ to the small ribosomal subunit can take place (for reviews see ref. [2–4]). Very little about this mechanism in eukaryotic microorganisms such as yeast is known.

Recently, a protein synthesizing system prepared from cell-free yeast extract was described [5] which under appropriate conditions was capable of binding radioactive methionine to the small ribosomal subunit and to components sedimenting in the 5 s area. From this it was reasoned that Met-tRNA$_i$ was bound via an eIF-2 · GTP · Met-tRNA$_i$ complex although the extracts did not stringently respond to GTP [6]. This paper presents more detailed data about the requirements for Met-tRNA$_i$ binding to initiation factor and 40 s ribosomal subunits, using a partially fractionated system containing exclusively yeast components.

Materials and Methods

Yeast strain and culturing conditions

The tetraploid strain 2200 of *S. cerevisiae* [7] was cultured in medium described elsewhere [8]. Cells were harvested at 3.6×10^8 cells per ml, washed and finally suspended in buffer I (100 mM Tris-HCl, pH 7.8, 100 mM KCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 3% v/v glycerol).

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Preparation of ribosomes

Unless otherwise stated, all preparations were carried out between 0 °C and 4 °C.

Suspended cells were homogenized for 1 min in a Braun homogenizer under CO_2-cooling. The homogenate was subsequently centrifuged at $40000 \times g$ for 20 min. The pellet was discarded and the supernatant was layered on top of a 34% w/w sucrose cushion containing buffer II (30 mM Tris-HCl, pH 7.4, 50 mM KCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol) and centrifuged 18 h at $250000 \times g$. The supernatant was concentrated and extensively dialyzed against buffer II. This fraction was taken as a source for aminoacyl synthetase. The ribosomal pellets were suspended in buffer II. For ribosomal subunit isolation the KCl concentration was raised to 500 mM by the addition of 4 M KCl to the ribosome suspension. 6000 A_{260} units of ribosomes were layered on sucrose density gradient no. 263 [9] containing buffer II but 500 mM KCl. Centrifugation was carried out for 16 h at 28000 rpm in Beckman's Ti 15 zonal rotor. Separated subunits were identified photometrically at 260 nm. Pooled fractions containing 40 s subunits were again centrifuged for 18 h at $250000 \times g$. The pellets were suspended in buffer II and stored frozen until use

Preparation of initiation factors

Suspended ribosomes were adjusted to 400 A_{260} units per ml and 4 M KCl was added dropwise to

give 0.5 M KCl. This suspension was stirred for one hour. Then the ribosomes were sedimented by centrifugation for 5 h at 361 000 × g. The supernatant was concentrated by ammonium sulphate (75% saturation) and subsequent dialysis against buffer III (20 mM Tris-HCl, pH 7.4, 200 mM KCl, 10 mM 2-mercaptoethanol, 10% v/v glycerol). This fraction was called ribosomal KCl wash.

For further fractionation 40 mg of the ribosomal KCl wash was applied to a heparin-Sepharose column (1 × 8 cm) prepared as described [10] which was equilibrated with buffer III. After washing the column with buffer III its KCl concentration was raised in steps of 100 mM KCl up to finally 500 mM KCl. Fractions of each elution step containing UV absorbing material were combined, concentrated and dialyzed against buffer III. Proteins which eluted at 200 mM KCl were designated as 200 mM KCl fraction, those eluting between 200 mM KCl and 300 mM KCl as 300 mM KCl fraction etc.

Preparation of Met-tRNA$_i$

The initiator tRNA was isolated according to [11] out of crude yeast tRNA (Boehringer). Enriched tRNA$_i$ was charged in a reaction mixture containing 30 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 2 mM ATP, 100 A_{260} units/ml tRNA, 6 µM [^{35}S]methionine (1 to 5 Ci/mmol) and 0.4 mg/ml protein of supernatant fraction mentioned in chapter "preparation of ribosomes". After incubation (20 min, 30 °C), the tRNA was recovered by phenol extraction and subsequent ethanol precipitation.

When the Met-tRNA synthetase activity of various protein fractions was tested, in a 100 µl reaction mixture only 1 A_{260} unit crude tRNA and 500 pmol [^{35}S]methionine was offered. After completion of the reaction, TCA precipitable radioactive material retained on glass fiber filters was counted as described in the next chapter.

Millipore filter assays

Binding of [^{35}S]Met-tRNA$_i$ to proteins was assayed under the following conditions, if not otherwise stated: A 100 µl reaction mixture contained 20 mM Tris-HCl, pH 7.4, 80 mM KCl, 10 mM 2-mercaptoethanol, 0.5 mM GTP and 20 pmol [^{35}S] Met-tRNA$_i$. The reaction was started with protein fraction in amounts as indicated in the legends. The samples were incubated for 4 min at 30 °C, thereafter, they were immediatly diluted (50-fold) with cold buffer of the same salt composition as the reaction mixture and passed through Millipore filters (HA 0.45 µm). The radioactivity retained on the filters was measured by liquid scintillation technique using a PPO-toluene cocktail.

Sucrose density gradient experiments

After the first incubation described in the previous chapter, the reaction mixture was supplemented with 1 A_{260} unit 40 s ribosomal subunits, 0.05 A_{260} units AUG codon and various amounts of $MgCl_2$ (see legends). These additions raised the volume of the reaction mixture to 110 µl. The samples were incubated a second time for 5 min at 0 °C. Afterwards, glutaraldehyde was added to give 0.1%, a concentration which does not produce artifacts [12]. The samples were layered on top of a linear sucrose density gradient (10% w/w to 30% w/w) made up in buffer (20 mM Tris-HCl, pH 7.4, 80 mM KCl, 10 mM 2-mercaptoethanol and $MgCl_2$ as in the reaction mixtures) and were centrifuged for 4.5 h, 10 °C, 41 000 rpm in a SW 41 rotor (Beckman). The gradient was fractioned (~ 480 µl/fraction), 5 ml of Beckman's Sucrose Solve cocktail was added to each fraction and the radioactivity was counted in a liquid scintillation counter (Beckman LS 7000).

Results

Identification of eIF-2 activity in the ribosomal KCl wash

The ability of the ribosomal KCl wash to bind Met-tRNA$_i$ to the small ribosomal subunit was taken as a criterion that it contained eIF-2 activity. The KCl wash fraction was first incubated with GTP and [^{35}S]Met-tRNA$_i$ but without Mg^{2+} to form complexes between Met-tRNA$_i$ and eIF-2. Thereafter, the reaction mixtures were supplemented with AUG, various concentrations of $MgCl_2$ and 40 s ribosomal subunits and incubated a second time to allow reaction between complexes and 40 s particles. The reaction was stopped by fixation with glutaraldehyde to a concentration of 0.1%. Subsequently, the reaction mixtures were layered on top of a sucrose density gradient. After centrifugation the distribution of radioactivity was analysed. Fig. 1 reveals that maximum binding of Met-tRNA$_i$ to 40 s

particles was achieved in the presence of 3 mM $MgCl_2$. The control experiments show that no spontaneous binding between Met-tRNA$_i$ and 40 s subunits took place in absence of KCl wash fraction.

It was desirable to evaluate parameters of the reaction displayed during the first incubation by the Millipore filter technique. Since a considerable amount of Met-tRNA synthetase activity was detected in the KCl wash fraction (data not shown), which might cause retention of radioactive Met-tRNA$_i$ on Millipore filters, further fractionation was necessary.

Chromatography on heparin-Sepharose

Waldman *et al.* [13] and van der Mast *et al.* [10] reported that initiation factors from reticulocytes and Krebs II ascites cells respectively bind to heparin-Sepharose.

The ribosomal KCl wash was applied to a heparin-Sepharose column and eluted with buffers containing 200 mM KCl in steps of 100 mM KCl. Fractions of each eluting step in which protein was detected were combined and tested for their ability to bind [^{35}S] Met-tRNA$_i$ to 40 s ribosomal subunits similar to the experiments depicted in Fig. 1. From Fig. 2 it is seen that only the 400 mM KCl fraction could catalyze the binding of appreciable amounts of Met-tRNA$_i$ to 40 s ribosomal subunits, whereas the other fractions revealed only greatly reduced binding activities. In preliminary experiments, those protein concentrations of each fraction yielding maximum binding had been estimated.

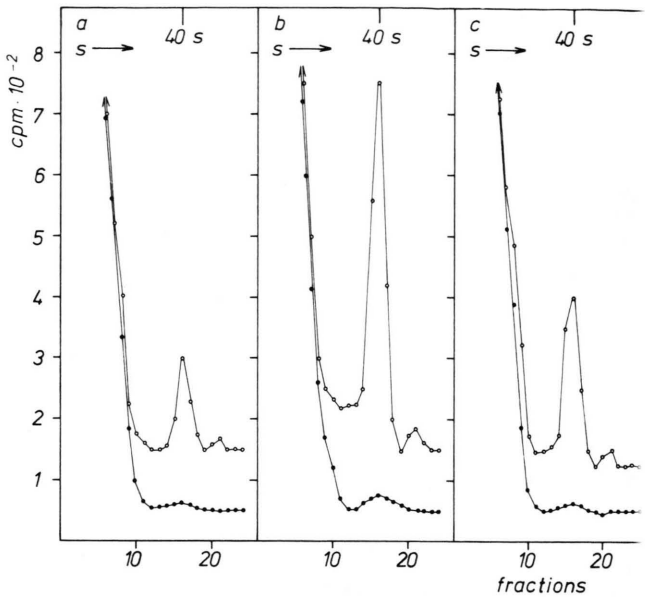

Fig. 1. Binding of Met-tRNA$_i$ to 40 s ribosomal subunits in presence ○—○ or absence ●—● of KCl wash fraction (73 µg) at (a) 1 mM $MgCl_2$, (b) 3 mM $MgCl_2$ and (c) 5 mM $MgCl_2$ in the second incubation. For centrifugation conditions and incubations see, "Materials and Methods"!

Subsequently, the same amounts of each fraction as assayed in the experiments depicted in Fig. 2 were tested in two different ways for their capability (1) to charge tRNA with [^{35}S]methionine and (2) to cause retention of [^{35}S]Met-tRNA$_i$ on Millipore filters. From Table I it is seen that the 200 mM KCl fraction contained the bulk synthetase activity but

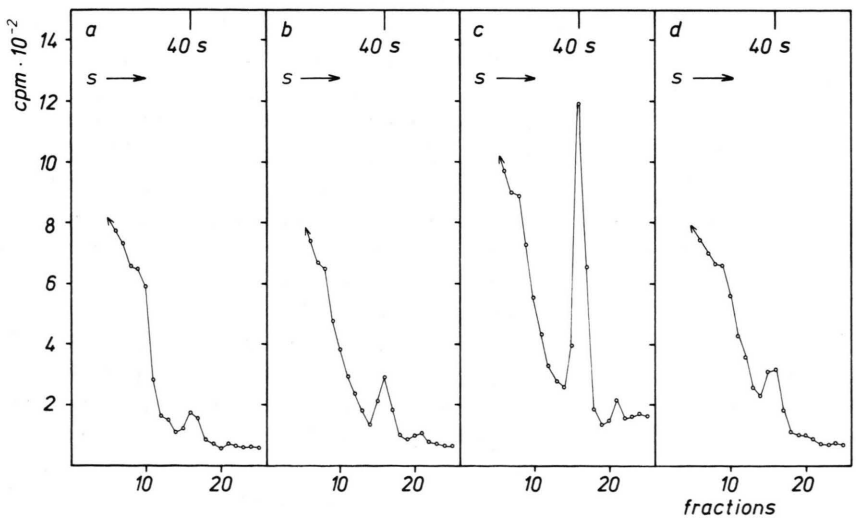

Fig. 2. Binding of Met-tRNA$_i$ to 40 s ribosomal subunits catalyzed by various fractions eluted from the heparin-Sepharose column. (a) 200 mM KCl fraction (42 µg), (b) 300 mM KCl fraction (32 µg), (c) 400 mM KCl fraction (23 µg), (d) 500 mM KCl fraction (30 µg). The second incubation was carried out at 3 mM $MgCl_2$, centrifugation conditions as described under "Materials and Methods"!

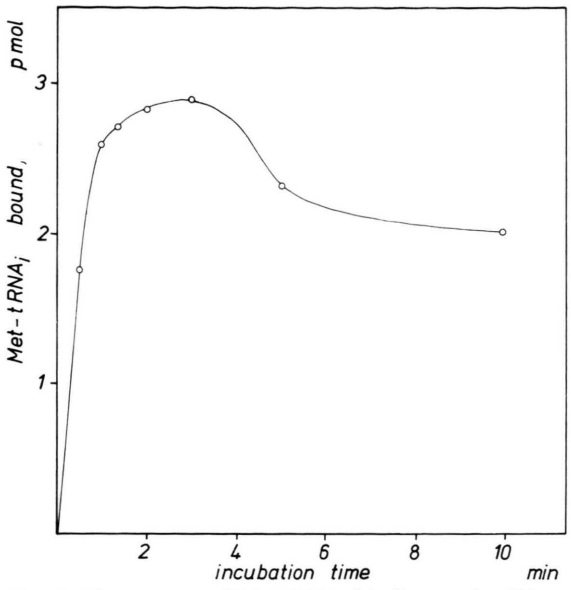

Fig. 3. Time course of Met-tRNA$_i$ binding to the 400 mM KCl fraction (23 µg). Incubation were carried out as described in "Materials and Methods"!

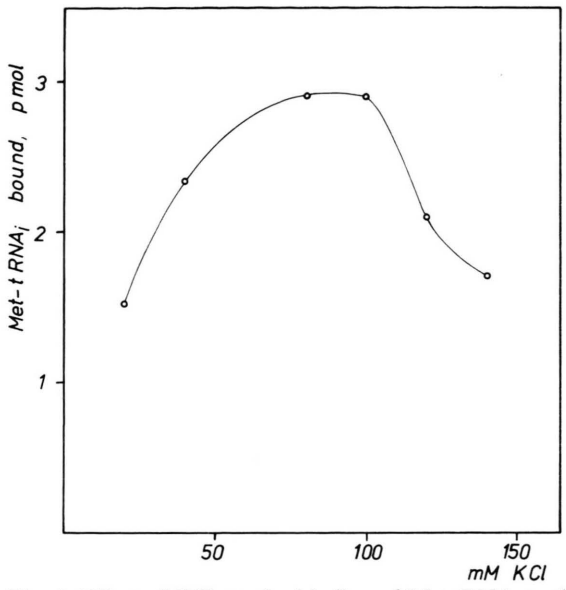

Fig. 4. Effect of KCl on the binding of Met-tRNA$_i$ to the 400 mM KCl fraction (23 µg). Incubations were carried out as described under "Materials and Methods"!

almost no complexes were retained on Millipore filters. In the following fraction the synthetase activity gradually dropped towards zero. The 400 mM KCl fraction yielded the highest binding of Met-tRNA$_i$ measured with the Millipore filter technique. A fraction of this activity was also found in the 300 mM KCl and 500 mM KCl fractions. These results obtained by the Millipore filter technique were consistent with those of sucrose density gradient experiments illustrated in Fig. 2 which supported the assumption that Met-tRNA$_i$ was retained on Millipore filters by the action of eIF-2. Methionine-tRNA synthetase seemed to form no complexes with Met-tRNA$_i$ that were detectable on Millipore filters. The explanation for the sliding elution of synthetase might be that heparin posses different kinds of protein binding sites leading to a complex elution behaviour.

Table I. Met-tRNA$_i$ binding activity and Met-tRNA synthetase activity in various fractions eluted from the heparin-Sepharose column. Incubations were carried out as described under "Materials and Methods".

Fraction	µg	pmol Met-tRNA$_i$ retained on Millipore filters	pmol Met-tRNA formed
200 mM KCl	42	0.08	18
300 mM KCl	32	0.6	14
400 mM KCl	23	2.9	5
500 mM KCl	30	0.5	0.7

Conditions of Met-tRNA$_i$ binding to the 400 mM KCl fraction measured by the Millipore filter technique

As seen from Fig. 3, binding of Met-tRNA$_i$ proceeded very quickly at 30 °C. The reaction was complete after 3 min. Longer incubation times led to dissociation of complexes. Fig. 4 shows the effect of K^+ on Met-tRNA$_i$ binding. In further experiments the optimal concentration of 90 mM KCl was applied.

In Fig. 5 complex formation in dependence of various concentrations of the 400 mM KCl fraction in presence of 0.5 mM GTP or its absence is demonstrated. There was only small stimulation by GTP at factor concentrations yielding maximum Met-tRNA$_i$ binding. High concentrations of protein even reduced the stimulatory effect.

Additions of various amounts of GTP caused only maximal stimulation of 34% at 330 µM GTP in a broad optimum (Fig. 6). Presence of an energy preserving system (phosphoenol pyruvat, pyruvat kinase) did not change the characteristics (data not shown). As already mentioned in the "Introduction",

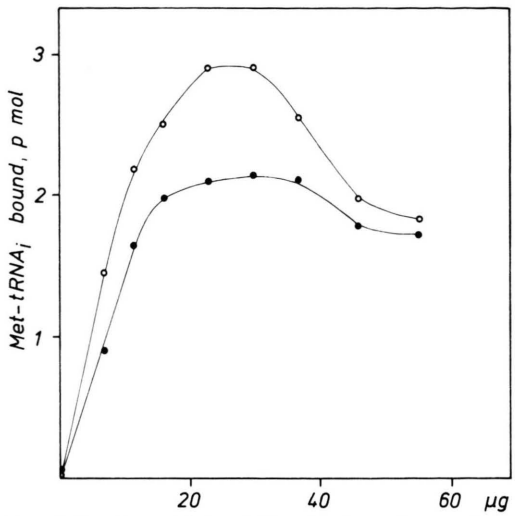

Fig. 5. Binding of Met-tRNA$_i$ in dependence of concentration of the 400 mM KCl fraction in presence ○—○ or absence ●—● of 0.5 mM GTP. Incubations were carried out as described under "Materials and Methods" except that 90 mM KCl was introduced.

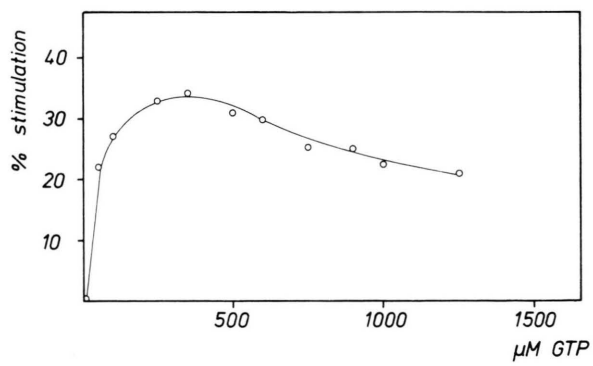

Fig. 6. Influence of GTP to Met-tRNA$_i$ binding to the 400 mM KCl fraction (23 µg). Incubations were carried out as described under "Materials and Methods" except that 90 mM KCl was introduced.

it was reported that binding of methionine to components of the yeast cell-free extract did not significantly respond to GTP [6]. eIF-2 isolated out of ascites tumor cells also did not reveal GTP dependent binding of Met-tRNA$_i$ [14], whereas the reticulocyte initiation system can undergo multiple stimulation by GTP [15].

Upon addition of Mg^{2+} to the reaction mixture, complex formation was inhibited either in presence or absence of GTP (Fig. 7). This Mg^{2+} lability of complexes was also observed with eIF-2 of other sources [15, 16].

Dependencies in the formation of the 40 s initiation complex

Although GTP did not stimulate Met-tRNA$_i$ binding to proteins in the 400 mM KCl fraction, it is possible that GTP might play an essential role in the formation of the 40 s initiation complex. To test this assumption, complexes of [^{35}S]Met-tRNA$_i$ and the 400 mM KCl fraction were preformed in absence or presence of GTP under otherwise optimal conditions in two-fold reaction mixtures. Before a second incubation, the reaction mixtures were supplemented with 40 s ribosomal subunits, $MgCl_2$ (3 mM) and in some experiments with AUG codons. After the second incubation (5 min, 0 °C), one half of each sample was fixed by glutaraldehyde and submitted

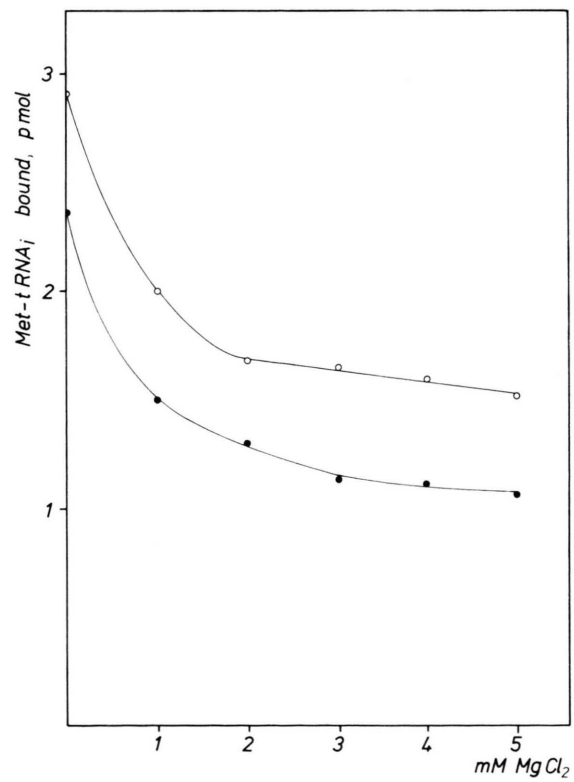

Fig. 7. Inhibition of complex formation between Met-tRNA$_i$ and 400 mM KCl fraction (23 µg) by $MgCl_2$ in presence ○—○ or absence ●—● of 330 µM GTP. Incubations were carried out as described under "Materials and Methods" except that 90 mM KCl was introduced.

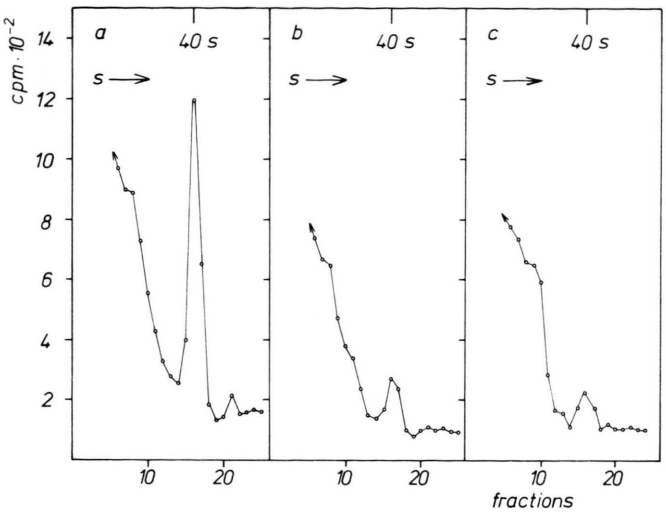

Fig. 8. Binding of Met-tRNA$_i$ to 40 s ribosomal subunits catalyzed by the 400 mM KCl fraction (23 µg) when (a) AUG and 330 µM GTP were present, (b) 330 µM GTP was present but AUG was omitted and (c) GTP was omitted and AUG was present at 3 mM $MgCl_2$ in the second incubation. For centrifugation conditions and incubations, see "Materials and Methods".

to density gradient centrifugation, the other half was diluted with cold buffer and passed through Millipore filters.

The analysis of the gradient clearly revealed that no binding of Met-tRNA$_i$ to 40 s ribosomal subunits took place in absence of GTP or AUG codon (Fig. 8). Table II however demonstrates that, compared to the controls, 40 s subunits slightly reduce the amount of Met-tRNA$_i$ retained on Millipore filters. When 40 s subunits were present, AUG codons and GTP had almost no effect. From this it was concluded that the Met-tRNA$_i$ binding activity of the 400 mM KCl fraction was not dependent on 40 s subunits and/or AUG codon. The relevance of these experiments will be discussed in the next chapter.

Table II. Influence of AUG, GTP and 40s ribosomal subunits on the retention of [^{35}S-]Met-tRNA$_i$ on Millipore filters when the 400 mM KCl fraction (23 µg) was present. The incubations were carried out as described in the legend to Fig. 8 except that after the second incubation the fixation was omitted but the sample were diluted and passed through Millipore filters.

System	pmol Met-tRNA$_i$ retained on filters
+ AUG; + GTP; − 40 s	1.4
− AUG; + GTP; − 40 s	1.4
+ AUG; − GTP; − 40 s	1.1
+ AUG; + GTP; + 40 s	1.0
− AUG; + GTP; + 40 s	1.0
+ AUG; − GTP; + 40 s	0.9

Discussion

Different translational initiation factors which could bind Met-tRNA$_i$ or its formylated form had been found in the ribosomal KCl wash, as well as in the cytoplasm of various eukaryotic cell types [1, 10, 14−26]. One of these was called eIF-2, which exclusively binds Met-tRNA$_i$ or its formylated derivate and GTP. The regulatory function of eIF-2 is well established [2−4], in contrast to eIF-2A (IF-M1) or eIF-2A like initiation factors which were reported to bind fMet-tRNA$_f$, acetyl-Phe-tRNA or Phe-tRNA to 40 s ribosomal subunits in a GTP independent but template dependent reaction [21−25]. The activity of the latter factors could only be measured by the Millipore filter technique indicating the lability of these complexes in comparison to those formed by eIF-2 which are stable even during density gradient centrifugation. With eIF-2A or similar factors, retention of labelled tRNA on Millipore filters only occurred when ribosomal particles were present in the reaction mixture [21−25].

It is the question now, whether the Met-tRNA$_i$ binding activity of the fractions isolated out of the yeast ribosomal KCl wash was due to eIF-2 or to eIF-2A like factor(s). This question becomes more urgent since binding of Met-tRNA$_i$ could not be stimulated by GTP greatly in the yeast system (Fig. 6) although eIF-2 activity found in other sources could usually be stimulated by GTP [1, 10, 15−20] except in eIF-2 preparations of ascites tumor cells [14]. The results of Table II clearly showed that retention of

Met-tRNA$_i$ on Millipore filters was not dependent on 40 s ribosomal subunits either in presence or absence of GTP and AUG codon. This means that residual activity due to the lack of ribosomal subunits could not be the reason for the radioactivity retained on Millipore filters in the experiments corresponding to Figs. 3–7 and Table I. Furthermore, stable complexes of the small ribosomal subunit and Met-tRNA$_i$ could be detected in a sucrose density gradient as a result of a AUG and GTP dependent reaction (Fig. 8). Although many authors reported template independent binding of Met-tRNA$_i$ to the small ribosomal subunit by the action of eIF-2 [1, 10, 14–17, 19, 20] this mechanism is not generally accepted [18, 26]. In light of this information it is very unlikely that binding of Met-tRNA$_i$ in the reactions presented here was due to a factor other than eIF-2. The GTP-dependency of Met-tRNA$_i$ binding to the small ribosomal subunit hints at the participation of GTP in the binding of Met-tRNA$_i$ by yeast eIF-2. To clear the nature of this mechanism, further investigations are necessary.

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