INHIBITION OF INITIATION FACTOR eIF-2 ACTIVITY FROM *ARTEMIA SALINA* EMBRYOS BY TRANSFER RNAs

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1. Introduction

The eukaryotic initiation factor, which promotes the GTP-dependent binding of initiator Met-tRNA$_i$ to the small ribosomal subunit, is referred to as eIF-2 according to a recently accepted nomenclature [1]. eIF-2 also makes a ternary complex with GTP (or non-hydrolyzable GTP analogs) and eukaryotic initiator Met-tRNA$_i$ in the absence of ribosomes [2–11]. eIF-2 can discriminate against eukaryotic and prokaryotic non-initiator aminoacyl tRNAs and against prokaryotic initiator Met-tRNA$_f$ whether formylated or not [2,8,10,11].

It has previously been reported that the inclusion of an excess of uncharged (deacylated) tRNAs in assay mixtures did not inhibit ternary complex formation promoted by eIF-2 from either rabbit reticulocytes [3,7] or L cells [4]. During the course of our study on the mechanism of action of eIF-2, we have observed that, as assayed by ternary complex formation, the activity of *Artemia salina* eIF-2 is strongly inhibited by an excess of unfractionated tRNA preparations and purified tRNA species. In contrast to the specificity of *A. salina* eIF-2 for eukaryotic Met-tRNA$_i$ in the formation of ternary complex [10,11], both prokaryotic and eukaryotic non-initiator tRNAs inhibit ternary complex formation. The implications of these findings are discussed.

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2. Materials and methods

eIF-2 was prepared from high-salt ribosomal washes of developing *A. salina* cysts by gel filtration on Sephadex G-200 followed by chromatography on DEAE-cellulose and finally CM–Sephadex chromatography, according to the procedure described by us [11]. eIF-2 at all three stages of purification was used in the present study. Initiator [^{35}S]Met-tRNA$_i$ was prepared by acylation of unfractionated rabbit or calf liver tRNA with [^{35}S]methionine using *E. coli* aminoacyl-tRNA synthetase [11,12]. All preparations of [^{35}S]Met-tRNA$_i$ were passed through Sephadex G-50 columns. Unfractionated rabbit liver and calf liver tRNA were purchased from General Biochemicals and Boehringer Mannheim, respectively. Other materials were obtained from the following companies: GTP from P.-L. Biochemicals: GMP–P(NH)P 5′-guanylylimidodiphosphate) from ICN; unfractionated yeast tRNA from Boehringer Mannheim and unfractionated *E. coli* tRNA from Schwarz. Unfractionated *A. salina* tRNA was prepared from the postribosomal supernatant fraction by phenol extraction followed by chromatography on Sephadex G-100 [10]. Pure yeast tRNA$_f^{Ala}$ (spec. act. 1750 pmol/A_{260} unit), yeast tRNA^{Ile} (spec. act., 800 pmol/A_{260} unit), *E. coli* tRNA^{Ser} (spec. act. 888 pmol/A_{260} unit) and *E. coli* tRNA^{Gly} (~20% pure) were kindly provided by Drs O. Bhanot and R. W. Chambers of our department. Purified *E. coli* tRNA$_f^{Met}$ was a generous gift of Dr N. K. Gupta, University of Nebraska. eIF-2 was assayed by ternary complex formation as follows. Unless mentioned otherwise, complete reaction mixtures (0.1 ml) contained 20 mM Tris–HCl buffer,

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Table 1
Effect of unfractionated rabbit liver tRNA on ternary complex fromation

Expt. No.	tRNA addition	[^{35}S]Met-tRNA$_i$ bound (pmol)	Inhibition (%)
1	None	0.64	–
	+	0.25	61
2	None	0.75	–
	+	0.12	84

Ternary complex fomation was assyed with GTP as described under Materials and methods, using 2.0 pmol calf liver [^{35}S]Met-tRNA$_i$ (33 427 cpm/pmol; expt. 1) and 3.8 pmol rabbit liver [^{35}S]Met-tRNA$_i$ (3360 cpm/pmol; expt. 2). 20 μg and 2.6 μg eIF-2, at the DEAE-cellulose and CM-Sephadex stages of purification, were used in expt. 1 and 2, respectively. 0.2 A_{260} units of unfractionated and uncharged rabbit liver tRNA was added, where indicated. Minus GTP blanks, with or without added tRNA, have been subtracted. For expt. 1, these blanks were 0.02 pmol and 0.08 pmol in the presence and absence of added tRNA, respectively. The corresponding blanks in expt. 2 were 0.02 pmol and 0.04 pmol.

pH 7.4, 1 mM dithiothreitol, 5% (v/v) glycerol, 150 mM Kcl, 2 pmol rabbit or calf liver, [^{35}S]Met-tRNA$_i$, 0.1 mM GTP, eIF-2 and, where indicated, uncharged tRNA. eIF-2 was added last and incubation was for 10 min at 30°C. 2 ml of ice-cold buffer (filtration buffer) containing 20 mM Tris–HCl, pH 7.4 and 150 mM KCl was then added and the diluted reaction mixtures were passed through millipore filters (HAWPO 2500; 0.45 μM) under suction. The filters were then washed three times with 2 ml ice-cold filtration buffer. The filters were dried and counted in a Beckman Liquid scintillation counter.

3. Results

The effect of addition of unfractionated rabbit liver tRNA on ternary complex formation is shown in table 1. eIF-2, at the DEAE-cellulose and CM–Sephadex stages of purification, were used in expt. 1 and 2, respectively. It may be seen that, in both experiments, ternary complex formation is inhibited by the addition of unfractionated rabbit liver tRNA.

The inhibition of ternary complex formation is dependent on the concentration of uncharged tRNA. The results obtained with unfractionated rabbit liver tRNA and CM–Sephadex eIF-2 are shown in table 2. Under the assay conditions, significant inhibition is observed at an input of 0.05 A_{260} units of tRNA and complex formation is almost completely inhibited when the concentration of unfractionated rabbit liver tRNA is raised to 0.4 A_{260} units/0.1 ml assay mixture.

Inhibition of ternary complex formation is also observed by the addition of unfractionated tRNAs from sources other than rabbit liver. The results are given in table 3. Each unfractionated tRNA preparation (tested at an input of 0.2 A_{260} unit) was found to be inhibitory, although the magnitude of the inhibitory effect was variable depending on the source of the tRNA. Similar results were obtained using eIF-2 at the CM–Sephadex stage of purification (data not shown) It may also be seen from the results given in table 3 that, regardless of the source of the unfractionated tRNA, the per cent inhibition of ternary complex

Table 2
Effect of different concentrations of unfractionated rabbit liver tRNA on ternary complex formation

Rabbit liver tRNA added (A_{260} units)	[^{35}S]Met-tRNA$_i$ bound (pmol)	Inhibition (%)
None	0.58	–
0.05	0.44	24
0.10	0.27	53
0.20	0.07	88
0.40	0.03	95

Conditions similar to those of expt. 2, table 1. The concentration of unfractionated rabbit liver tRNA was varied as indicated. A minus GTP blank of 0.04 pmol has been substracted. The specific radioactivity of rabbit liver [^{35}S]Met-tRNA$_i$ was 3200 cpm/pmol.

Table 3
Effect of unfractionated tRNAs from different sources on ternary complex formation

tRNA added	Nucleotide added	[^{35}S]Met-tRNA$_i$ bound (pmol)	Inhibition (%)
None	GTP	0.65	–
Yeast	GTP	0.28	57
E. coli	GTP	0.38	42
A. salina	GTP	0.17	74
None	GMP–P(NH)P	1.27	–
Yeast	GMP–P(NH)P	0.93	27
E. coli	GMP–P(NH)P	1.02	20
A. salina	GMP–P(NH)P	0.51	60

Conditions similar to those of expt. 1, table 1. 01 mM GMP–P(NH)P or GTP was used, as indicated. 0.2 A_{260} units of each unfractionated tRNA preparation was added, where indicated. A blank (0.09 pmole), without any added nucleotide, has been subtracted. The specific radioactivity of calf liver [^{35}S]Met-tRNA$_i$ was 33 27[illegible] cpm/pmol.

formation by tRNA is higher when GTP (rather than GMP–P(NH)P) is used. However, it is important to note that, relative to GMP–P(NH)P, less ternary complex is formed with GTP under these conditions [11].

The effect of unfractionated rabbit liver tRNA on ternary complex formation, using two concentrations of eIF-2 and either GTP or GMP–P(NH)P, is shown in table 4. In the absence of rabbit liver tRNA, similar amounts of ternary complex (around 0.5 pmol) were formed with GMP–P(NH)P and GTP using 25 μl and 50 μl of eIF-2, respectively. Furthermore, under these conditions, the inhibition of ternary complex formatio[illegible]

Table 4
Effect of unfractionated rabbit liver tRNA on ternary complex formation at different eIF-2 concentrations

tRNA addition	Nucleotide added	eIF-2 added (μl)	[^{35}S]Met-tRNA$_i$ bound (pmol)	Inhibition (%)
None	GTP	25	0.25	–
+	GTP	25	0.02	92
None	GTP	50	0.49	–
+	GTP	50	0.15	70
None	GMP–P(NH)P	25	0.53	–
+	GMP–P(NH)P	25	0.13	75
None	GMP–P(NH)P	50	1.06	–
+	GMP–P(NH)P	50	0.44	58

Ternary complex formation was assayed as described under Materials and methods, using 0.2 mM GMP–P(NH)P or GTP, 100 mM KCl and 2 pmol calf liver [^{35}S]Met-tRNA$_i$ (30 740 cpm/pmol). Indicated aliquots of eIF-2 (1.65 mg/ml), at the Sephadex G-200 stage of purification, were added. 0.2 A_{260} units of unfractionated rabbit liver tRNA was added, where indicated. Appropriate minus nucleotide blanks, with or without added tRNA, have been subtracted. These blanks were: 0.03 pmol (25 μl eIF-2, no tRNA); 0.06 pmol (50 μl eIF-2, no tRNA); 0.01 pmol (25 μl eIF-2, plus tRNA) and 0.02 pmol (50 μl eIF-2, plus tRNA).

Table 5
Effect of purified tRNA species on ternary complex formation

Expt. No.	tRNA added	[^{35}S]Met-tRNA$_i$ bound (pmol)	Inhibition (%)
1	None	0.47	–
	Yeast tRNA^{Ala}	0.16	66
	E. coli tRNA^{Gly}	0.31	34
	Crude yeast tRNA	0.23	51
	Crude *E. coli* tRNA	0.29	38
2	None	0.43	–
	Yeast tRNA^{Ile}	0.29	33
	E. coli tRNA^{Ser}	0.16	63
3	None	0.47	–
	E. coli tRNA$_f^{Met}$	0.37	21
4	None	0.55	–
	Yeast tRNA^{Ala}	0.10	82
	E. coli tRNA^{Ser}	0.08	85

Conditions similar to those of table 4, using 0.2 mM GMP–P(NH)P. 0.3 mM Mg^{2+} was present in all reaction mixtures. Approximately 0.2 A_{260} units of each tRNA preparation was added, where indicated. 41.3 μg and 2.6 μg of eIF-2, at the Sephadex G-200 and CM-Sephadex stages of purification, were used in expt. 1–3 and expt. 4, respectively. Appropriate minus GMP–P(NH)P blanks, with or without added tRNA, have been substracted. These blanks ranged between less than 0.01–0.03 pmol. The specific radioactivity (cpm/pmol) of calf liver [^{35}S]Met-tRNA$_i$ was 29 280 (expt.1), 26 315 (expt.2), 17 780 (expt.3) and 19 192 (expt.4).

by rabbit liver tRNA was about the same (70–75%) using either GTP or GMP–P(NH)P. In contrast, the per cent inhibition is significantly different when the effect of unfractionated tRNA is studied under conditions in which different amounts of ternary complex are formed in the absence of tRNA (e.g., 92% inhibition using 25 μl eIF-2 and GTP versus 58% inhibition using 50 μl eIF-2 and GMP–P(NH)P). The results indicate that, regardless of whether GTP or GMP–P(NH)P is used, the per cent inhibition by unfractionated tRNA is relatively low when relatively high amounts of ternary complex are formed in the absence of tRNA.

The results given in table 5 provide evidence that purified tRNA species also inhibit ternary complex formation. eIF-2 at the Sephadex G-200 and CM–Sephadex stages of purification were used in expt. 1–3 and expt. 4, respectively. Approximately 0.2 A_{260} unit of each tRNA preparation was added. Pure yeast tRNA^{Ala} is strongly inhibitory (expt. 1,4). The per cent inhibition obtained with pure yeast tRNA^{Ala} is somewhat greater than that obtained with crude unfractionated yeast tRNA (expt. 1) Partially purified *E. coli* tRNA^{Gly} (~20% pure) gave about the same inhibition as crude unfractionated *E. coli* tRNA (expt. 1) Yeast tRNA^{Ile} (about 50% pure) was less inhibitory than pure yeast tRNA^{Ala}, whereas *E. coli* tRNA^{Ser} (about 50% pure) was more inhibitory than *E. coli* tRNA^{Gly} (see expt. 1,2). It should also be noted that the degree of inhibition caused by yeast tRNA^{Ala} is about the same as that due to *E. coli* tRNA^{Ser}, while *E. coli* tRNA$_f^{Met}$ is less inhibitory than *E. coli* tRNA^{Ser}.

Highly purified eIF-2 (chromatographed on CM–Sephadex) appears to be more sensitive to inhibition by tRNA species (expt. 4; also see table 1).

4. Discussion

It is known that *A. salina* eIF-2 forms a GTP-dependent ternary complex with eukaryotic initiator Met-tRNA$_i$ only [10,11]. In contrast, ternary complex

formation is inhibited by prokaryotic and eukaryotic non-initiator tRNAs (table 5) as well as by poly-r[U] and poly-r[A] (data not shown). A possible explanation for these apparently contradictory findings is the following. The binding of GTP (or GMP–P(NH)P) to eIF-2 may trigger a conformational change such that the eIF-2 · GTP (or eIF-2 · GMP–P(NH)P) complex can subsequently recognize and bind only eukaryotic Met-tRNA$_i$. On the other hand, free eIF-2, having a different conformation, may not exhibit this specificity. Thus, free eIF-2 may bind other RNAs like prokaryotic and eukaryotic non-initiator tRNAs, specially when these RNAs are present in reaction mixtures at relatively high concentrations. Under these conditions, a sizable fraction of the added eIF-2 would be tied up as eIF-2 · RNA complexes which may be non-functional in ternary complex formation. It should be pointed out in this connection that Safer et al. [7] have presented kinetic evidence which suggests that ternary complex formation proceeds via an ordered reaction sequence in which the binding of GTP to reticulocyte eIF-2 precedes the binding of Met-tRNA$_i$. These investigators have observed that the binary complex eIF-2 · Met-tRNA$_i$ cannot subsequently bind GTP.

In contrast to our results with *A. salina* eIF-2, it has been reported that ternary complex formation promoted by rabbit reticulocyte [3,7] and L cell [4] eIF-2 is not inhibited by unfractionated tRNA preparations from reticulocytes and partially tRNA$_m^{Met}$ and tRNA$_f^{Met}$ from rat liver, respectively. One explanation for this discrepancy is that the RNA-binding properties of reticulocyte and L cell eIF-2 are different from those of *A. salina* eIF-2. However, it should also be noted that, in the reticulocyte and L cell systems [3,4,7], the effect of addition of uncharged tRNA preparations was tested under conditions in which relatively large amounts (1.7–2.5 pmol) of ternary complex were formed. Our results (tables 3,4 and other data not shown) indicate that the per cent inhibition by tRNA preparations is relatively low under conditions which permit the formation of relatively high amounts of ternary complex. In addition, we have observed that some tRNAs are less inhibitory than others (table 5). It remains to be seen whether those tRNAs, which strongly inhibit ternary complex formation in the *A. salina* system, are also inhibitory in other systems when tested under our experimental conditions.

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