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A Supernatant Factor Involved in Initiation Complex Formation with Eukaryotic Ribosomes

(*Artemia salina*/embryos/cysts/brine-shrimp eggs/elongation factors)

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ABSTRACT **Embryos of the brine shrimp, *Artemia salina*, were used in a study of polypeptide chain initiation in an *in vitro* system from a eukaryote. A protein, isolated from the high-speed supernatant, has been highly purified and shown to have properties that suggest it is the eukaryotic equivalent of the *Escherichia coli* initiation factor F_2: It promotes the AUG-dependent binding of fMet-tRNA (*E. coli*) to the *Artemia* 40S ribosomal subunit, but not to either the 60S or 80S species; the bound fMet-tRNA is placed in a site on the smaller subunit from which it reacts with puromycin upon addition of the 60S subunit; and the activity is sensitive to aurintricarboxylic acid and edeine, specific inhibitors of initiation. The factor, a basic protein of molecular weight about 100,000, is inactivated by *N*-ethylmaleimide, an SH-binding reagent, and is clearly distinct from the *Artemia* elongation factors, T_1 and T_2. In addition, the factor stimulates the poly(U)-dependent binding of Phe-tRNA (*E. coli*) to the *Artemia* 40S ribosomal subunit. This reaction, though similar to the fMet-tRNA-binding reaction, differs in that the bound Phe-tRNA is largely resistant to release by puromycin.**

In some eukaryotic organisms protein synthesis is initiated by unformylated Met-tRNA$_F$ (1) and, as described for a reticulocyte system, initiation may involve protein factors that, like the bacterial initiation factors, are loosely bound to the ribosomes (2–4). However, the mechanism of polypeptide chain initiation in eukaryotes is still poorly understood. For further studies of eukaryotic initiation, we have chosen the embryos of the brine shrimp (*Artemia salina*) which, as shown by Hultin and Morris (5), have ribosomes that are competent for poly(U) translation. After encapsulation in a hard shell, *Artemia* embryos remain viable upon drying; the dehydrated cysts, commercially available as "brine shrimp eggs", consist mainly of embryos at the gastrula stage of development (6). The ready availability of this material makes it eminently suited for work involving the isolation and purification of eukaryotic initiation factors. Thus, we decided to look for an AUG-dependent binding of Met-tRNA$_F$ to *Artemia* cyst ribosomes. However, since we were unable to find in the cysts a tRNA^{Met} species that could be formylated by *Escherichia coli* transformylase, a property common to most of the initiating Met-tRNA$_F$ species isolated from eukaryotic sources (1), we used *E. coli* fMet-tRNA as substrate.

The experimental design included (*a*) use of the small, 40S subunits, (*b*) low Mg^{2+} concentration, and (*c*) an incubation temperature of 0°C. These conditions were selected because (*a*) initiation in *E. coli* involves the small, 30S ribosomal subunit (7), (*b*) low Mg^{2+} concentration increases the requirement for initiation factors (7), and (*c*) *E. coli* ribosomes show good AUG-dependent binding of fMet-tRNA at 0°C with F_2 as the only initiation factor, but negligible binding at 25°C unless F_1 is also present (8). Condition (*c*) thus narrowed down the immediate aim to a search for an F_2-like factor.

Preliminary trials at high Mg^{2+} concentration (10–15 mM) and temperature (25°C) showed the occurrence of a "nonenzymatic", AUG-dependent fMet-tRNA and, to a lesser extent, Met-tRNA binding to 40S subunits. Most of the bound fMet was available for fMet-puromycin synthesis and, therefore, seemed to be on an initiation site. At 2.5 mM Mg^{2+} and 0°C there was no binding in either the presence or absence of a 1.0 M-KCl ribosomal wash. However, addition of a small amount of the solution from which the ribosomes were originally pelleted promoted binding.

We report on the properties of a supernatant factor required for the AUG-dependent binding of fMet-tRNA$_F$, as well as the poly(U)-mediated binding of Phe-tRNA to the small, 40S ribosomal subunit. This factor, which is distinct from the elongation factors T_1 and T_2, may be a eukaryotic equivalent of the bacterial initiation factor F_2. Similar soluble factors have been recently reported in rat liver (9)* and yeast†.

MATERIALS AND METHODS

Ribosomes and Ribosomal Subunits. All operations are conducted at 0–4°C. *Artemia* cysts are washed and ground essentially as described (10). In a typical preparation, 30 g of cysts (dry weight) are suspended in 100 ml of ice-cold 1% NaClO, stirred for 5 min, and then diluted with 1 liter of ice-cold distilled water. Upon standing, the cysts rapidly sediment from the suspension leaving behind in the supernatant damaged unviable organisms and other debris. The supernatant is poured off and the sediment is washed 10 times, each time with about 400 ml of water to ensure removal of the hypochlorite. The cysts are washed finally in 200 ml of a buffer (buffer A) containing 35 mM Tris·HCl (pH 7.4)–70 mM KCl–9 mM magnesium acetate–0.1 mM EDTA–10 mM 2-mercaptoethanol–0.25 M sucrose, sedimented, resuspended in about 100 ml of buffer A, and finally ground in a mortar. After centrifugation of the suspension for 15 min at 10,000 rpm in the Sorvall SS34 rotor to remove debris, nuclei, and mitochondria, the supernatant is centrifuged for 15 min at 30,000 rpm in the Spinco no. 30 rotor to free the preparation from glycogen, which is present in large amounts and would otherwise sediment with the ribosomes. The supernatant is finally centrifuged at 50,000 rpm for 90 min in the Spinco Ti 60 fixed-angle rotor (150,000 × *g*). The ribosomal pellet, which consists exclusively of 80S ribosomes (Fig. 1*A*), is used for the preparation of the subunits. The surface of the clear, glassy pellet is washed gently with a high-salt buffer (buffer B), containing 50 mM Tris·HCl (pH 7.8)–700 mM KCl–11 mM magnesium acetate–20 mM 2-mercaptoethanol, and then suspended in

Abbreviation: DTT, dithiothreitol.

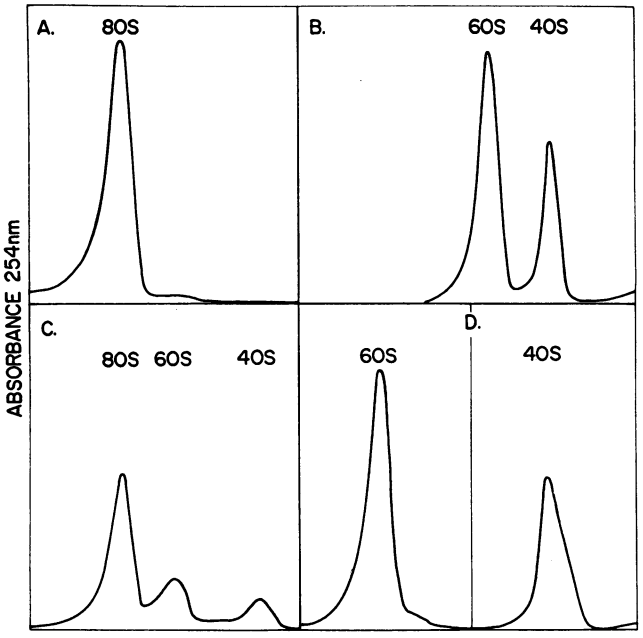

FIG. 1. Sucrose density gradient centrifugation analysis of *A. salina* ribosomes and ribosomal subunits. Suitable aliquots were layered on 5 ml of a 15–30% sucrose gradient in the appropriate buffer and centrifuged as indicated at 4°C. The gradients were analyzed in an Isco (model D) gradient analyzer. (A) "Native" ribosomes: 0.7 A_{260} unit of ribosomal pellet in 40 mM Tris·HCl (pH 7.75)–70 mM KCl–7 mM magnesium acetate, centrifuged in the Spinco SW39 rotor for 135 min at 39,000 rpm. (*B*) Dissociation of 80S ribosomes in high salt: 0.9 A_{260} unit of ribosomal pellet in buffer B (without 2-mercaptoethanol), centrifuged as in *A*. (*C*) Reconstitution of 80S ribosomes: 0.2 A_{260} unit of 40S and 0.48 A_{260} unit of 60S subunits mixed in the same buffer and centrifuged as in *A*. (*D*) Isolated subunits: 0.5 A_{260} unit of 40S or 0.7 A_{260} unit of 60S subunits in 90 mM Tris·HCl (pH 7.4)–140 mM KCl–8 mM magnesium acetate–0.5 mM DTT, separately centrifuged in the Spinco SW65 Ti rotor for 80 min at 50,000 rpm.

buffer B to a concentration of 400 A_{260} units/ml. 1 ml of this suspension is layered on 50 ml of a 15–30% sucrose gradient in buffer B and centrifuged in the Spinco SW25.2 rotor at 24,000 rpm for 13–14 hr at 4°C. 80S ribosomes completely dissociate into 60S and 40S subunits under these conditions (Fig. 1*B*). Fractions comprising the faster-sedimenting 40% of the 60S peak and the slower-sedimenting 70% of the 40S peak are pooled and *immediately* diluted with 1 volume of 20 mM 2-mercaptoethanol–30 mM magnesium acetate. The subunits are concentrated to a clear pellet by centrifugation for 10 hr at 40,000 rpm and 4°C in the Spinco Ti 60 rotor. The pellets are gently suspended in buffer A with a teflon pestle and, after addition of 1 volume of glycerol, stored at −20°C. They retain full activity in poly(U) translation for several months. As assayed by poly(U) translation, the 40S subunits show less than 2.5%, and the 60S subunits less than 5%, contamination with the other species. Zonal centrifugation of the ribosomal subunits also demonstrates low cross-contamination (Fig. 1*D*). When an equimolar mixture of subunits is suspended in a buffer similar to buffer A and analyzed, extensive reassociation to 80S ribosomes takes place (Fig. 1*C*).

Washed 80S ribosomes are prepared from the crude ribosomal pellets. The pellets are washed gently with a buffer containing 50 mM Tris·HCl (pH 7.4)–500 mM KCl–20 mM 2-mercaptoethanol, and suspended in this buffer to a concentration of 200 A_{260} units/ml, stirred for 20 min at 4°C, and repelleted at 60,000 rpm in a Spinco 65 fixed-angle rotor. The pellets are suspended and stored as described for the subunits. They are used for assay of elongation factors T_1 and T_2.

Supernatant Factor. The factor is purified from the S-150 supernatant by fractionation with ammonium sulfate, followed by chromatography on DEAE-cellulose, phosphocellulose, and Sephadex G-200; it is quite stable when kept at −20°C in 50% glycerol. Under the conditions of the standard assay (see below), 1 mg promotes the binding of about 1000 pmol of fMet-tRNA and 600 pmol of Phe-tRNA. The corresponding values for the S-150 supernatant are 6 and 3 pmol/mg of protein, respectively. The purification will be described in detail in a subsequent publication.

Assays. The standard assay of the supernatant factor is based on its requirements for the ApUpG (AUG)-dependent binding of fMet-tRNA$_F$, or the poly(U)-dependent binding of Phe-tRNA, to *Artemia* 40S ribosomal subunits. Samples contain, in a volume of 0.06 ml, 80 mM Tris·HCl buffer (pH 7.4), 150 mM KCl, 2.5 mM magnesium acetate, 0.2 mM GTP, 2 mM dithiothreitol (DTT), 0.6 A_{260} of 40S ribosomal subunits, supernatant factor, and either 0.1 A_{260} AUG and 15 pmol *E. coli* f[^{14}C]Met-tRNA (480 cpm/pmol) or 1 A_{260} poly(U) and 17 pmol *E. coli* [^{14}C]Phe-tRNA (830 cpm/pmol), After incubation for 30 min at 0°C, the reaction is stopped by the addition of 3.0 ml of an ice-cold buffer containing 50 mM Tris·HCl (pH 7.75)–60 mM KCl–5 mM magnesium acetate, and the ribosome-bound radioactivity is determined by the Millipore filtration procedure of Nirenberg and Leder (11). Filters are dried under an infrared lamp and the retained radioactivity is measured in Omnifluor (New England Nuclear Corp.) in a Packard Tri-Carb liquid scintillation spectrometer. ^{14}C is counted at 90% efficiency.

The assay of chain elongation factor T_1 (aminoacyl-tRNA-binding factor) is based on its requirement for the poly(U)-

TABLE 1. *Inactivation of supernatant factor by N-ethylmaleimide (NEM)*

Incubation	Factor activity			
	fMet-tRNA		Phe-tRNA	
	Binding to 40S (pmol)	Inhibition (%)	Binding to 40S (pmol)	Inhibition (%)
Factor alone	1.02	0	0.53	0
Factor				
+ NEM (1.4 mM)	0.53	48	0.14	74
+ NEM (6.0 mM)	0.33	68	0.08	85
+ NEM (10.8 mM)	0.27	74	0.07	87
+ DTT (50 mM)				
+ NEM (10.8 mM)	1.27	0	0.54	0

Factor (5.2 μg) was incubated in a solution containing, in 65 μl, 66 mM Tris·HCl buffer (pH 7.2), 143 mM KCl, 3 mM magnesium acetate, and NEM at the indicated concentrations. After incubation for 10 min at 24°C, unreacted NEM was inactivated with excess (50 mM) DTT and factor activity was measured on 10-μl aliquots by the standard assay. The last sample contained DTT throughout the incubation period. Net binding values are given, blanks without factor, of 0.18 and 0.05 pmol for fMet-tRNA and Phe-tRNA, respectively, were subtracted.

dependent binding of Phe-tRNA by 80S ribosomes (12). T_1 only weakly stimulates the binding of Phe-tRNA to 40S subunits. Samples contain, in a volume of 0.06 ml, 40 mM Tris·HCl buffer (pH 7.85), 120 mM KCl, 5 mM magnesium acetate, 1.25 mM GTP, 2 mM DTT, 0.37 A_{260} 0.5 M KCl-washed 80S ribosomes, 1 A_{260} poly(U), 17 pmol *E. coli* [^{14}C]Phe-tRNA (same as in the preceding assay), and a source of T_1. After incubation for 30 min at 22°C, the ribosome-bound radioactivity is measured as above.

The assay for elongation factor T_2 (translocase) is based on its requirement for the poly(U)-dependent synthesis of polyphenylalanine ([^{14}C]Phe incorporation into heat-stable, acid-insoluble material) from [^{14}C]Phe-tRNA by 80S ribosomes, in the presence of T_1. The composition of the samples is exactly as in the T_1 assay, with T_1 present in excess. After incubation for 15 min at 22°C, the reaction is terminated by the addition of 3 ml of 5% trichloroacetic acid. The samples are then heated to 90°C for 15 min, cooled, and filtered through Millipore membranes. The filters are dried and the retained radioactivity is measured as above.

Aminoacyl-puromycin synthesis is assayed according to Leder and Bursztyn (13), as described in the legend to Table 5, and is stopped by the addition of 1 ml of either cold 0.1 M sodium acetate (pH 5.5) (for *N*-acyl-aminoacyl-puromycin), or 2.0 M $(NH_4)_2CO_3$ (pH 9.0) (for aminoacyl-puromycin). After the addition of 1.5 ml of ethyl acetate and vigorous shaking for 1–2 min, the phases are separated by low-speed centrifugation. 1 ml of the ethyl acetate layer is counted in a scintillation vial containing 10 ml of Bray's (14) solution.

Miscellaneous. Phe-tRNA, Lys-tRNA, and fMet-tRNA labeled with ^{14}C in the phenylalanine, lysine, and methionine residues, respectively, were prepared by acylation of crude *E. coli* W tRNA (Schwarz BioResearch) and formylation of the Met-tRNA$_F$ species with *E. coli* Q13 high-speed supernatant (S-150) as a source of aminoacyl-tRNA synthetases and transformylase. Reaction mixtures contained, in a final volume of 2.0 ml, 100 mM Tris·HCl buffer (pH 7.8), 10 mM KCl, 10 mM magnesium acetate, 5 mM ATP, 4 mM DDT, about 0.05 mM of either [^{14}C]phenylalanine (455 Ci/mol), [^{14}C]lysine (312 Ci/mol), or [^{14}C]methionine (265 Ci/mol), 280 A_{260} of *E. coli* tRNA, *E. coli* Q13 S-150 supernatant (3 mg protein), and, in the case of methionine, 150 μg of N^5-formyltetrahydrofolic acid (Leucovorin, American Cyanamid Co.). After incubation for 20 min at 37°C, the mixture was extracted with freshly distilled phenol equilibrated with 10 mM sodium acetate buffer (pH 5.5). The aqueous layer was then applied to a column (0.9 × 45 cm) of Sephadex G-25 (Pharmacia, medium grade), equilibrated with the sodium acetate buffer, and eluted with the same buffer. The fractions of highest radioactivity that emerged in the excluded volume were pooled. The charging ratio was about 40 pmol/A_{260} unit for Phe-tRNA, 17 pmol/A_{260} unit for Lys-tRNA, and 45–55 pmol/A_{260} unit for Met-tRNA. As determined by the acceptance ratio of [^{14}C]formate to [^{3}H]methionine, the ratio tRNA$_F^{Met}$ to total tRNA^{Met} was about 0.7. The formyl donor [^{14}C]N^{10}-formyltetrahydrofolic acid was prepared according to Rabinowitz (15), with a purified (16) enzyme fraction from *Artemia* cysts as a source of formyltetrahydrofolate synthetase.

For some experiments, highly purified f[^{14}C]Met-tRNA$_F$ was prepared from *E. coli* tRNA$_F^{Met}$ with a methionine-accepting capacity of 1413 pmol/A_{260}, kindly supplied by Dr. G. D. Novelli, Oak Ridge National Laboratory. The preparation was as described for crude tRNA, except for the use of a tRNA-free S-150 fraction from *E. coli* Q13 as a source of methionyl-tRNA synthetase and transformylase. This fraction was prepared by dialysis of the standard S-150 preparation against 0.2 M Tris·HCl buffer (pH 7.4)–10 mM 2-mercaptoethanol, and application of the dialyzed solution (20 mg of protein) to a column (1.2 × 17 cm) of DEAE-cellulose (Whatman, DE 52) equilibrated with the same buffer. Transformylase and methionyl-tRNA synthetase activities are not retained and are recovered in the effluent. This enzyme fraction contained less than 0.1 pmol of tRNA^{Met} per mg of protein. The crude formylmethionyl-tRNA preparation will be designated fMet-tRNA, whereas the highly purified preparation will be referred to as fMet-tRNA$_F$.

Protein was determined by the Lowry method (17), with bovine serum albumin as a standard. ApUpG, UpUpU, and ApApA were products of the Miles Laboratories, GpUpG was a gift of Dr. M. W. Nirenberg (to Dr. R. Mazumder of this laboratory). The source of brine-shrimp eggs (10), and other methods and preparations were described (18).

RESULTS

Properties of Factor. The supernatant factor is a basic protein of molecular weight about 100,000. To ascertain whether SH groups are essential for its activity, the factor was preincubated with various concentrations of N-ethylmaleimide (NEM) under conditions similar to those used by Mazumder *et al.* (19) for the bacterial initiation factor F_2. Table 1 shows that 10.8 mM *N*-ethylmaleimide causes about 80% inactivation. However, contrary to the findings with F_2 (19), GTP failed to afford protection against inhibition by this reagent. We also find that omission of GTP from incubation mixtures is without apparent effect on the binding reaction.

Properties of Binding Reaction. At low Mg^{2+} concentrations (2.5–3.0 mM), AUG-dependent ribosomal binding of fMet-tRNA is observed at 0°C but not at 25°C, whereas poly(U)-directed binding of Phe-tRNA is obtained at either temperature. This may be due to the fact that the AUG–40S–fMet-tRNA complex is less stable than is the poly(U)–40S–Phe-tRNA complex. When F_2 is the only initiation factor present, the AUG-directed binding of fMet-tRNA to *E. coli* ribosomes is high at 0°C, but very low at 25°C (8). At higher Mg^{2+} concentrations, e.g., 10 mM, factor-dependent formation of an AUG–40S–fMet-tRNA complex is observed at 25°C, but the net factor effect is less than at 0°C. Little or no 40S binding of Phe-tRNA or Lys-tRNA is detected at 0°C with UUU or AAA as messenger, whether in the absence or presence of supernatant factor, but GUG is effective in mediating factor-dependent binding of fMet-tRNA.

The factor-dependent binding reaction, whether with fMet-tRNA or Phe-tRNA, occurs solely on 40S subunits. There is no reaction with 80S ribosomes or, as shown in Table 2, with an equimolar mixture of 40S and 60S subunits (line 2 of Expts. 1, 2, and 3), probably due to their association to 80S couples under the conditions of the assay. As further seen in Table 2 (Expt. 1, line 3), the elongation factor T_1 is without effect on the binding of fMet-tRNA$_F$ to either 40S or 40S + 60S subunits. However, in the case of Phe-tRNA, although T_1 is moderately effective with 40S (Expt. 2, line 3), it promotes considerable binding to 40S + 60S subunits (Expts. 2 and 3, line 3; Expt. 4). These results indicate that the supernatant factor is different from elongation factor T_1. As shown in Fig.

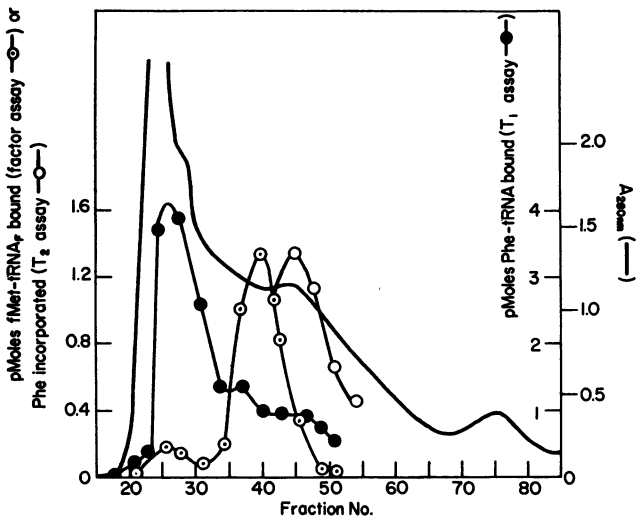

FIG. 2. Separation of *Artemia* factor, T_1, and T_2 activities by gel filtration. An ammonium sulfate fraction (30–70% saturation) from the S-150 supernatant was applied to a Sephadex G-200 column (1.5 × 85 cm) equilibrated with a buffer containing 50 mM Tris·HCl (pH 7.2)–150 mM KCl–0.1 mM EDTA–2.0 mM DTT, and eluted with the same buffer at 4°C. 1.5-ml fractions were collected and suitable aliquots were assayed for each activity as described in *Methods*. The aliquots used for each assay were: factor, 20 μl; T_1, 15 μl; T_2, 10 μl, in the presence of 5 μl of a pool of fractions *24–25* as a source of T_1.

2, the factor can in fact be readily separated from both T_1 and T_2 by chromatography on Sephadex G-200. Moreover, as seen

TABLE 2. *Subunit specificity of factor-dependent binding reaction*

Expt. no.	Additions: Aminoacyl-tRNA	Factor (μg)	T_1 (μg)	Aminoacyl-tRNA binding (pmol) with: 40S	40S + 60S
1	fMet-tRNA$_F$	None	None	0.37	0.73
		1.04	None	1.71 (1.34)	0.73 (0.00)
		None	50	0.40 (0.03)	0.74 (0.01)
		1.04	50	1.87 (1.50)	0.75 (0.02)
2	Phe-tRNA	None	None	0.09	0.18
		1.04	None	0.53 (0.44)	0.24 (0.06)
		None	50	0.35 (0.26)	5.83 (5.65)
3	Phe-tRNA	None	None	0.08	0.21
		1.67	None	0.76 (0.68)	0.27 (0.06)
		None	50*	—	5.58 (5.37)
4	Phe-tRNA	None	None	—	0.10
		None	50	—	3.27 (3.17)

Conditions were as in the standard assay, except that samples containing Tris·HCl, KCl, Mg^{2+}, GTP, DTT, and 40S subunits (0.6 A_{260} unit), with or without 60S subunits (1.47 A_{260} units), were incubated for 10 min at 0°C. The reaction mixtures were then completed (aminoacyl-tRNA being the last addition), and incubation was continued for a further 30 min at 0°C (Expts. 1 and 4) or 20 min at 24°C (Expts. 2 and 3). The concentration of Mg^{2+} was 8 mM throughout. Values in parentheses are net binding values promoted by factor and/or T_1 addition. T_1 was derived from a pool of fractions *26–29* of Fig. 2.

* Fusidic acid (0.065 mM) was included with T_1 in this sample to prevent synthesis of polyphenylalanine that might occur were traces of T_2 present.

TABLE 3. *Effect of supernatant factors on poly(U) translation by Artemia ribosomes*

Factor additions	[^{14}C]Phenylalanine polymerized (pmol)
T_1 (13 μg)	0.32
T_2 (15 μg)	0.22
T_1 + T_2	1.49
Factor (1 μg)	0
+ T_1	0.42
+ T_2	0.23

Conditions are those of the T_2 assay (see *Methods*). The source of T_1 was fraction *25*, that of T_2 fraction *47* of Fig. 2. A blank of 0.15 pmol, in the absence of added factors, was subtracted.

in Table 3, in the poly(U)directed synthesis of polyphenylalanine from Phe-tRNA, which requires both T_1 and T_2, neither elongation factor can be rep'aced by the supernatant factor.

The binding reaction is inhibited by inhibitors of initiation. As seen in Table 4, low concentrations of aurintricarboxylic acid or edeine inhibit 40S binding of both fMet-tRNA and Phe-tRNA. At low concentrations, aurintricarboxylate is a specific inhibitor of initiation in both prokaryotic and eukaryotic organisms (20), and edeine blocks the initiator site on the small, 30S ribosomal subunit in *E. coli* (21).

In the *E. coli* system, the synthesis of fMet-puromycin in the absence of G factor is taken as proof that initiation factors place the initiator aminoacyl-tRNA directly in the P (peptidyl) or an equivalent site on the ribosome (7). It was, therefore, of interest to determine whether aminoacyl-tRNA bound to the *Artemia* 40S subunit under the influence of the supernatant factor is capable of forming aminoacyl-puromycin upon addition of the 60S subunit and in the absence of elongation factors. It is clear from Table 5 that this is indeed the case for fMet-tRNA$_F$ (Expts. 1 and 2), since 80–90% of the bound fMet- is available for fMet-puromycin synthesis. However, when the reaction is run under similar conditions with Phe-tRNA, none of the ribosomal-bound phenylalanine is puromycin reactive (Expt. 3), and only about 30% appears to be available for Phe-puromycin synthesis at 24°C (Expt. 4).

DISCUSSION

The binding reaction promoted by the *Artemia* supernatant factor, specifically, the AUG-dependent binding of fMet-

TABLE 4. *Effect of aurintricarboxylic acid (ATA) and edeine on factor-dependent aminoacyl-tRNA binding to 40S subunits*

Additions	Aminoacyl-tRNA binding (pmol): fMet-tRNA	Phe-tRNA
None	0.20	0.05
Factor (0.65 μg)	1.21	0.60
+ ATA (0.2×10^{-4} M)	0.72	0.04
+ ATA (1.0×10^{-4} M)	0.04	0.03
+ edeine (3.2×10^{-6} M)	0.07	0.03

ATA was commercial grade (Aluminon, Aldrich Chemical Co.) and edeine was a gift from Drs. W. Szer and Z. Kurylo-Borowska.

TABLE 5. *Puromycin reactivity of 40S-bound aminoacyl-tRNA*

Expt. no.	Additions to 1st incubation: aa-tRNA	Additions to 1st incubation: Subunit	Additions to 2nd incubation: Subunit	Aminoacyl-tRNA bound (pmol)	Aminoacyl-puro synthesized (pmol)	Aminoacyl-puro synthesized (% of bound)
1	fMet-tRNA	40S	None	1.24	0	0
		40S	60S	0.93	0.84	90
		None	60S	0	0	0
2	fMet-tRNA$_F$	40S	None	1.46	0	0
		40S	60S	1.00	0.78	78
		None	60S	0.01	0	0
3	Phe-tRNA	40S	60S	0.97	0	0
4	Phe-tRNA	40S	60S	1.31	0.4	31

Duplicate samples were first incubated for 30 min at 0°C under the conditions of the standard factor assay (except as noted below) with the indicated aminoacyl-tRNA and ribosomal subunit additions. Then they were incubated for a further 15 min at the same temperature with subunit additions as indicated. Just before the second incubation, one of the duplicate samples received puromycin (75 μg) and the other received an equal volume of water. The puromycin-treated sample measured the synthesis of aminoacyl-puromycin, the other sample determined ribosomal binding of aminoacyl-tRNA. Subunit additions: 40S, 0.6 A_{260} unit; 60S, 1.47A_{260} units. The values given are net values in the presence of factor (1.04 μg), blank values without factor (averaging about 20% of the net for aminoacyl-tRNA binding and 10% for aminoacyl-puromycin synthesis) having been subtracted. Deviations from the standard assay conditions were 167 mM KCl (Expts. 1 and 2) and 143 mM KCl (Expts. 3 and 4), as well as 7 mM Mg^{2+}, and incubation at 24°C in Expt. 4.

tRNA to the *Artemia* 40S ribosomal subunit, shares certain properties with the analogous reaction catalyzed in the *E. coli* systems by initiation factor F_2: (*a*) both reactions occur on the free, small ribosomal subunit, (*b*) both reactions exhibit similar sensitivity towards edeine and aurintricarboxylate, and (*c*) both factors induce the binding of fMet-tRNA on a site of the small ribosomal subunit that allows fMet-puromycin synthesis upon association with the large subunit. Although unlike F_2 the supernatant factor is a basic protein, it resembles F_2 in the following properties: (*a*) molecular weight about 100,000 (22), (*b*) inhibition by SH-binding reagents (19), and (*c*) promotion of AUG-dependent ribosomal binding of fMet-tRNA$_F$, detectable at 0°C but not at 25°C, in the absence of other initiation factors (8).

The isolation of initiation factors from 0.5 M-KCl washes of reticulocyte ribosomes has been reported (2, 3). Factor M_1 resembles the *Artemia* supernatant factor in not being retained by DEAE-cellulose, and both F_2 and the *Artemia* factor in its ability to stimulate AUG-dependent ribosomal binding of Met-tRNA or fMet-tRNA in the absence of other initiation factors. It remains to be seen whether, as we are inclined to believe, the aforementioned factors from rat liver (9)* and yeast† are in fact identical to the factor described here.

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