PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES XIX*: EIF-2† PROMOTES DISSOCIATION OF Met-tRNA$_f$·EIF-1·GTP COMPLEX AND Met-tRNA$_f$ BINDING TO 40S RIBOSOMES

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Received July 15, 1977

Summary

The peptide chain initiation factor, EIF-2 has been partially purified from the 0.5 M KCl ribosomal wash. The molecular weight of EIF-2 is approximately 450,000. The purified EIF-2 preparation promotes the dissociation of the ternary complex, Met-tRNA$_f$·EIF-1·GTP in the presence of Mg^{++} and is also required along with EIF-1 for AUG-directed Met-tRNA$_f$ binding to 40S ribosomes.

Several years ago, we reported (3-4) partial purification of three peptide chain initiation factors, EIF-1, EIF-2 and EIF-3, from the 0.5 M KCl wash of reticulocyte ribosomes. All three factors were required for maximum methionine transfer from Met-tRNA$_f$ into the terminal positions of the polypeptides synthesized in response to poly r (U-G) messenger. Later, we provided evidence that peptide chain initiation factor EIF-1 forms a ternary complex, Met-tRNA$_f$·EIF-1·GTP and that this ternary complex formation is the first step in peptide chain initiation (4-5). The presence of a Met-tRNA$_f$ binding factor similar to our EIF-1 and its role in the initial step in peptide chain initiation have also been reported by other laboratories (for a review, see Ref. 6).

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*Paper XVIII in this series is Ref. 1.

†In a recent meeting at the National Institutes of Health, Anderson and others proposed a uniform nomenclature for the eukaryotic peptide chain initiation factors(2). However, in view of the fact that there are many unanswered questions regarding the number of peptide chain initiation factors and their precise roles in the initiation process, the adoption of this nomenclature was left optional. Since none of the proposed factors are known to have Co-EIF-1 (see Ref. 10) and TDF (EIF-2) activities as described by our laboratory, we have used our own nomenclature for these factors.

According to the proposed nomenclature(2), our EIF-1 (Met-tRNA$_f$ binding factor) is eIF-2.

ISSN 0006-291X

Recently, we have noted (7) that Met-tRNA$_f$ binding activity (EIF-1) elutes from the DEAE-cellulose column in two forms; the ternary complexes formed with the earlier fractions (EIF-1A) are relatively stable in the presence of Mg^{++} whereas the ternary complexes formed with the latter fractions (EIF-1B) and eluting in the EIF-2 region dissociate extensively upon addition of Mg^{++}. Using hydroxylapatite column chromatography, we achieved a partial resolution of ternary complex dissociation activity (TDF, ternary complex dissociation factor) from Met-tRNA$_f$ binding factor (EIF-1) (8). We have interchangably used the term EIF-2 and TDF to describe this factor. Both EIF-1 and EIF-2 are required for maximum Met-tRNA$_f$ binding to 40S ribosomes in the presence of AUG codon (8). The peptide chain initiation factor EIF-3 catalyzes the association of Met-tRNA$_f$•40S•AUG complex to 60S ribosomes to form 80S initiation complex (9).

In this paper, we report further purification of EIF-2 using DEAE-cellulose chromatography and glycerol density gradient centrifugation. Upon density gradient centrifugation, EIF-2 sediments as a high molecular weight protein (approximate mol. wt., 450,000) and is well resolved from EIF-1 (mol. wt., 150,000 (8)). Purified EIF-2 preparations show very little Met-tRNA$_f$ binding activity and efficiently promote the dissociation of the ternary complex, Met-tRNA$_f$•EIF-1•GTP, formed with homogeneous preparations of EIF-1 in the presence of Mg^{++}. Both EIF-1 and EIF-2 are required for AUG-directed Met-tRNA$_f$ binding to 40S ribosomes.

Materials and Methods

The preparations of reticulocyte ribosomes, 40S ribosomal subunits and ribosomal 0.5 M KCl wash were the same as before (7-8). The peptide chain initiation factor, EIF-1, was purified to homogeneity following the procedure described previously (8). The homogeneous EIF-1 preparation showed three protein bands upon SDS-polyacrylamide gel electrophoresis corresponding to molecular weights of 60,000, 54,000 and 38,000.

Purification of EIF-2

The peptide chain initiation factor, EIF-2 was purified from the 0.5 M KCl ribosomal wash and the purification procedure up to the DEAE-cellulose step (Fraction III) was the same as described previously (7). As noted before (7), upon DEAE-cellulose chromatography, EIF-1 activity separates into two forms; the ternary complexes formed with the earlier fractions (EIF-1A) were resistant to Mg^{++} whereas the ternary complexes formed with the latter fractions (EIF-1B) and eluting in the EIF-2 region were extremely unstable in the presence of Mg^{++}. For EIF-2 preparation, the EIF-1B fractions were pooled and the pooled fractions were diluted with one volume of Buffer

D minus KCl (20 mM Tris-HCl, pH 8.0; 1 mM DTT; 50 μM EDTA and 10% glycerol) and were passed through a DEAE-cellulose column (DE-11) (1.3 cm x 7 cm) equilibrated with Buffer D minus KCl. The absorbed proteins were then eluted from the column with Buffer D containing 0.3 M KCl.

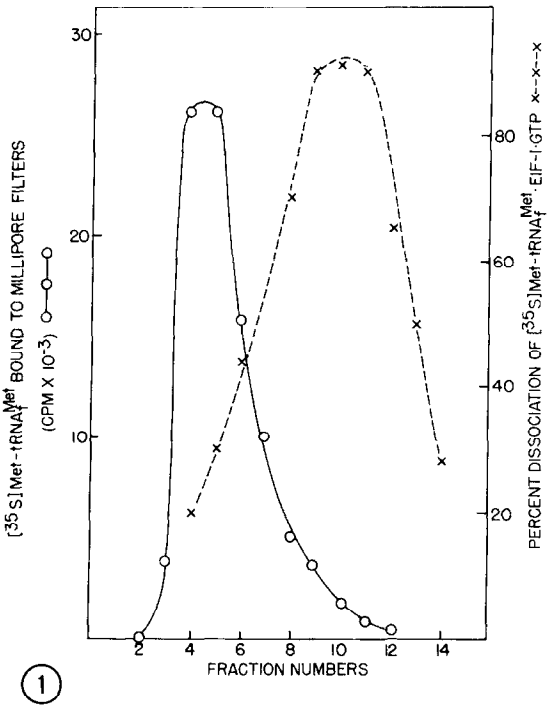
For density gradient fractionation, 0.2 ml aliquots of the concentrated protein fraction (approximately 4 mg per ml) were applied on top of 5 ml glycerol gradients (14 → 30%) containing 20 mM Tris-HCl, pH 8.0; 0.3 M KCl, 1 mM DTT and 50 μM EDTA. The solutions were then centrifuged at 45,000 rpm for 11 hours using a Spinco SW 50.1 rotor. The gradients were fractionated using an ISCO density gradient fractionator. A 50% glycerol displacing solution was used and 0.3 ml fractions were collected. Aliquots (0.025 ml) of the density gradient fractions were assayed for Met-$tRNA_f$ binding (EIF-1) activity following the procedure described previously (7). For TDF activity, the density gradient fractions were assayed for their abilities to dissociate Met-$tRNA_f$·EIF-1·GTP complex formed with homogeneous preparations of EIF-1 in the presence of Mg^{++}. The fractions showing TDF activity were pooled and concentrated as above by passage through a small DEAE-cellulose column and one-step elution with 0.3 M KCl. The concentrated TDF solution is stable at ice bath temperature for several weeks.

[^{35}S] Methionine (150-200 Ci/mmol) was purchased from Amersham/Searle and was diluted with unlabelled methionine to 5,000 to 10,000 cpm/pmol. Other materials and methods were the same as described previously (7).

Results

Fig. 1 describes the results of a typical glycerol density gradient fractionation of EIF-1 and TDF activities. The EIF-1 activity of the fractions was measured by ternary complex formation with [^{35}S] Met-$tRNA_f$ and GTP in the absence of Mg^{++}. For measurement of TDF activity, the ternary complexes were first formed in duplicates with homogeneous preparations of EIF-1 in the presence of different gradient fractions. The reaction mixtures were then mixed with either Mg^{++} (final concentration, 5 mM) or equal volume of water and the incubation continued at ice bath temperature for 15 minutes. The ternary complexes were then analyzed by Millipore filtration and the extent of dissociation of the ternary complexes in the presence and absence of Mg^{++} in each tube was compared. As shown in Fig. 1, the bulk of the TDF activity was clearly separated from EIF-1 activity; the TDF fractions, 10-12, showed negligible Met-$tRNA_f^{Met}$ binding (EIF-1) activity.

The results of the glycerol density gradient centrifugation were used to determine the approximate molecular weight of EIF-2. Several standard



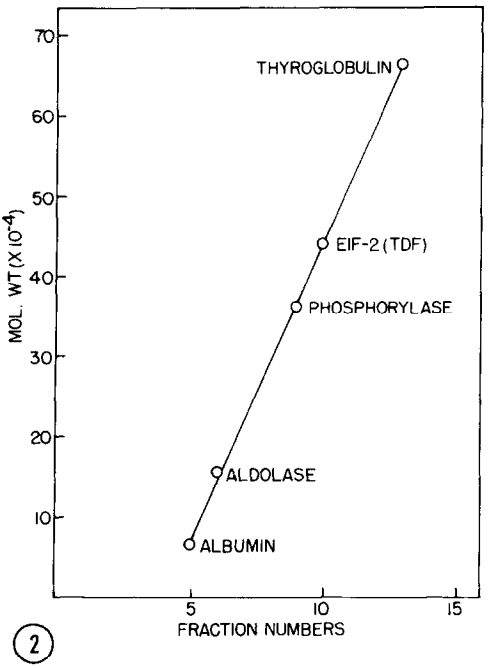


Fig. 1 Glycerol density gradient fractionation of EIF-1 and TDF (EIF-2) activities.

The procedure for glycerol density gradient separation of EIF-1 and TDF activities has been described under Materials and Methods. For determination of EIF-1 activity (o-o-o) standard Millipore filtration assay for ternary complex formation was used (7). A 0.025 ml aliquot of the gradient fraction was used in each experiment. The TDF activity was assayed using a two stage reaction. The conditions for Stage I were the same as were used for EIF-1 assay except that the reaction mixtures also contained 2.5 µg homogeneous EIF-1. The incubations were carried out in duplicate. At the end of Stage I, the solutions were mixed with either 5 µl, $MgCl_2$ (5 mM, final concentration) or H_2O and the incubations were continued at the ice bath temperature for additional 15 minutes. The reactions were then terminated by addition of 3 ml of cold washing buffer (20 mM Tris-HCl, pH 8.0; 100 mM potassium chloride). The washing buffers for the duplicate reactions containing 5 mM $MgCl_2$ also contained 5 mM $MgCl_2$. The solutions were then assayed by standard Millipore filtration. The percent dissociation of the ternary complexes (x---x---x) in each fraction was calculated from the radioactivity in the duplicate assay tubes with and without Mg^{++} added in the second stage of the experiment.

Fig. 2 Glycerol density gradient determination of molecular weight of TDF (EIF-2).

The molecular weight of TDF was determined by glycerol density gradient centrifugation. The density gradient centrifugation procedure has been described under Materials and Methods and the assay for TDF activity is the same as described in Fig. 1. Several standard proteins were used as markers. The peak A_{280} fractions of the marker proteins were plotted against their molecular weights. In several identical density gradient experiments, fraction 10 showed peak TDF activity.

TABLE I
TDF (EIF-2)-Catalyzed Dissociation of the Ternary Complex
($Met\text{-}tRNA_f^{Met}$•EIF-1•GTP) in the Presence of Mg^{++}

EIF-1 Added (µl)	TDF Added (µl)	Radioactivity Bound to Millipore Filters (pmol)	
		$-Mg^{++}$	$+Mg^{++}$
10	-	2.29	2.19
-	2	0.06	0.04
	5	0.12	0.04
10	2	2.32	0.90
10	5	2.66	0.47

Standard Millipore filtration assay conditions were used. Where indicated, EIF-1 (Fraction V, 0.5 mg per ml) (8) and TDF (glycerol gradient fraction; 0.5 mg per ml) were added.

proteins were used as markers. Fig. 2 shows a standard curve showing the molecular weights of the standards proteins vs their mobilities in the glycerol density gradient. The molecular weight of EIF-2 as determined by this method is approximately 450,000. Upon SDS-gel electrophoresis, this TDF preparation showed at least twelve bands ranging in molecular weights of 20,000 to 100,000 (not shown here) indicating that TDF is a protein complex made of several polypeptide chains.

The results presented in Table I describe the characteristics of TDF (EIF-2)-catalyzed dissociation of the ternary complex in the presence of Mg^{++}. As before, the ternary complexes were formed by incubation of homogeneous EIF-1 preparation, and where indicated EIF-2, at 37°. Mg^{++} (final concentration, 5 mM) was then added and the incubation continued at ice bath temperature. As shown in Table I, the ternary complex formed with the EIF-1 preparation is almost completely resistant to added Mg^{++}. The EIF-2 preparation alone has very little $Met\text{-}tRNA_f$ binding activity, and at a somewhat higher concentration (5 µl) stimulates $Met\text{-}tRNA_f$ binding to EIF-1, presumably due to the presence Co-EIF-1 activity in this preparation (10). However, when both EIF-1 and EIF-2 were added, the ternary complex was

TABLE II
Requirement of EIF-1 and EIF-2 for AUG-Directed

$Met\text{-}tRNA_f$ Binding to 40S Ribosomes

Factors Added	Radioactivity Bound to Millipore Filters (pmol)		
	-AUG -40S Rib	-AUG +40S Rib	+AUG +40S Rib
EIF-1	0.43	0.26	0.36
EIF-2	0.04	0.04	0.17
EIF-1 + EIF-2	0.42	0.45	1.20

Standard two stage Millipore filtration assay conditions were used (11). Where indicated, 5 µg EIF-1 and 2.5 µg of EIF-2 were added.

extensively dissociated upon addition of Mg^{++}. This dissociation reaction increased with increasing EIF-2 concentration; more than 80 percent dissociation was observed in the presence of 5 µl EIF-2.

The results presented in Table II clearly establish the requirement of both EIF-1 and EIF-2 for AUG-directed $Met\text{-}tRNA_f$ binding to 40S ribosomes. As noted above, we used a homogeneous preparation of EIF-1 and $Met\text{-}tRNA_f$ binding to 40S ribosomes was assayed using the two stage millipore filtration assay method (11). As shown in Table II, the homogeneous EIF-1 preparation by itself does not promote $Met\text{-}tRNA_f$ binding to 40S ribosomes in the presence or absence of AUG codon. The purified EIF-2 preparation showed small but significant $Met\text{-}tRNA_f$ binding to 40S ribosomes in the presence of AUG codon, presumably due to slight contamination of this EIF-2 preparation with EIF-1. However, when both EIF-1 and EIF-2 were added together, significant stimulation of $Met\text{-}tRNA_f$ binding to 40S ribosomes was observed and this stimulation of $Met\text{-}tRNA_f$ binding was almost entirely dependent upon added AUG codon.

Discussion

Upon glycerol density gradient centrifugation, the EIF-2 activity sediments as a high molecular weight protein complex and is well-resolved from the $Met\text{-}tRNA_f^{Met}$ binding (EIF-1) activity. This EIF-2 preparation promotes at least two reactions: (1) Dissociation of $Met\text{-}tRNA_f \cdot EIF\text{-}1 \cdot GTP$

in the presence of Mg^{++} and (2) Binding of Met-$tRNA_f$ to 40S ribosomes in the presence of AUG codon. It is not clear, at present, whether both activities reside in one protein or in different proteins. Upon SDS-gel electrophoresis, the EIF-2 preparation shows 12-14 bands corresponding to molecular weights ranging from 20,000 to 100,000. Apparently, EIF-2 is a complex composed of multiple polypeptide chains. Judged from its molecular composition (extremely high molecular weight and multiple polypeptide subunits), EIF-2 appears to be similar to EIF-3 of Staehelin et al (12-13), Issinger et al (14), and IF-3 of Thomson et al (15), although the precise catalytic roles of EIF-2, such as ternary complex dissociation activity and Met-$tRNA_f$ binding (mRNA dependent) to 40S ribosomes, have not yet been reported by other laboratories. Thomson et al report (15) that their IF-3 prevents the reassociation of ribosomal subunits and to a limited extent dissociates ribosomes into subunits.

The most significant aspect of this work is the demonstration of the requirements of mRNA and also two peptide chain initiation factor preparations, EIF-1 and EIF-2, for Met-$tRNA_f$ binding to 40S ribosomes. We have provided evidence that homogeneous EIF-1, which actively forms Met-$tRNA_f$•EIF-1•GTP, does not catalyze the binding of Met-$tRNA_f$ to 40S ribosomes with or without AUG codon (Table II). Another peptide chain initiation factor, EIF-2, is necessary in addition to EIF-1 for Met-$tRNA_f$ binding to 40S ribosomes and such binding is entirely dependent on added AUG codon.

The above results clearly differ from reports by other laboratories (12, 16-18) that in eukaryotic protein synthesis, unlike the prokaryotic system, the initiator tRNA binds to the 40S ribosomal subunit in the absence of messenger RNA, and EIF-1 alone can catalyze this binding reaction. Previously, we provided (11) several reasons for the discrepancies between our results and the results reported by other laboratories. For example, in some cases (16,18), Met-$tRNA_f$•40S complexes were fixed with cross-linking agents such as glutaraldehyde (18) or formaldehyde (16) and the complexes were then analyzed by density gradient centrifugation. We reported (11) that glutaraldehyde extensively degrades Met-$tRNA_f$•40S•AUG complexes and also increases background Met-$tRNA_f$ binding to 40S ribosomes, presumably by cross-linking Met-$tRNA_f$•EIF-1•GTP complex to non-specific proteins in 40S ribosomes. When analyzed by sucrose density gradient centrifugation, the radioactivity bound to 40S ribosomes in the absence of AUG codon was significantly increased in the presence of glutaraldehyde and the addition of AUG codon resulted in only marginal stimulation of Met-$tRNA_f$ binding. A similar observation has also been made by Smith et al (19) using formaldehyde as cross-linking agent.

However, we would like to emphasize that our results only demonstrate that, as in the E. coli. system, more Met-tRNA$_f$ is bound to 40S ribosomes when AUG codon is present, and do not provide any evidence as to which component, the initiator tRNA or mRNA, binds first to the 40S ribosomes. It is possible that either of these components (initiator tRNA or mRNA) may bind to 40S ribosomes first, but the complex becomes more stable when both components are present.

Finally, we have demonstrated that one of the factors, TDF (EIF-2) catalyzes the dissociation of the ternary complex in the presence of Mg^{++} and is also required for AUG-directed Met-tRNA$_f$ binding to 40S ribosomes. Apparently, the dissociation of the ternary complex is not inhibitory to peptide chain initiation and may possibly be an integral part of the overall initiation process. We believe that the TDF preparation promotes two reactions: (1) It facilitates the binding of mRNA to 40S ribosomes and also, (2) It promotes the dissociation of the ternary complex while this complex is bound to 40S or 80S ribosomes, thus releasing EIF-1 for recycling.

Acknowledgements

This research work was supported by NIH Research Grants GM-18796 and GM-22079. The authors thank Mr. Dennis Jurgens for the preparation of reticulocyte ribosomes and ribosomal salt wash.

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