PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES. XV: ISOLATION OF A RIBOSOMAL PROTEIN FACTOR (CO-EIF-1) WHICH STIMULATES Met-tRNA$_f^{Met}$ BINDING TO EIF-1*

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Summary

A protein factor, CO-EIF-1, has been partially purified from the high salt wash of reticulocyte ribosomes. CO-EIF-1, by itself, does not bind Met-tRNA$_f^{Met}$ but significantly stimulates (2-3 fold) Met-tRNA$_f^{Met}$ binding to EIF-1 and also increases the initial rate of this binding reaction. In the presence of CO-EIF-1, approximately 90 percent of the input EIF-1 (assuming a molecular weight of 140,000) was bound to Met-tRNA$_f^{Met}$.

Protein synthesis initiation in eukaryotic cells is a complex process and involves the participation of numerous protein factors (for a review, see Ref. 2). One of the most thoroughly studied factors is EIF-1 (3-14). This factor binds Met-tRNA$_f^{Met}$ in the presence of GTP to form a stable ternary complex, Met-tRNA$_f^{Met}$·EIF-1·GTP. We have previously reported (5) that EIF-1 can exist in at least two forms, EIF-1A and EIF-1B. The ternary complex formed with the highly purified factor, EIF-1A, is stable in the presence of Mg^{++}, whereas the same complex formed with the partially purified preparation dissociates extensively in the presence of 5 mM Mg^{++}. A protein factor present in the partially purified initiation factor preparation promotes the dissociation of the ternary complex in the presence of Mg^{++}.

Several laboratories have reported on the extensive purification of EIF-1. The molecular weight of this factor is approximately 140,000 (5,14). However, the specific activities (pmole of Met-tRNA$_f^{Met}$ bound per mg of protein) of near homogeneous EIF-1 preparations as reported from different laboratories are rather low; Majumdar et al., 2000 (6), Safer et al., 550 (11). These activities correspond to total bindings of input EIF-1 of only 28 (6) and 8 percent (11) respectively, assuming a 1:1 binding of EIF-1 to Met-tRNA$_f^{Met}$.

In this communication, we describe the partial purification and properties of a protein factor, CO-EIF-1 from the 0.5 M KCl ribosomal wash. This factor,

*Paper XIV in this series is Ref. 1.

Abbreviation: EIF-1, eukaryotic initiation factor 1.

by itself, does not bind Met-$tRNA_f^{Met}$, but significantly increases the initial rate and the total extent of Met-$tRNA_f^{Met}$ binding to purified EIF-1. In the presence of CO-EIF-1, approximately 90 percent of the input EIF-1 was bound to Met-$tRNA_f^{Met}$.

MATERIALS AND METHODS

Purification of EIF-1.

EIF-1 was prepared from the 0.5 M KCl ribosomal wash and the procedure up to the DEAE-cellulose step (Fraction III) was the same as described previously (6). The dialyzed fraction III was then passed through a phosphocellulose column (1 cm x 5 cm) equilibrated with Buffer D (5 mM Tris-HCl, pH 7.5; 0.1 M KCl; 1 mM dithiothreitol; 50 μM EDTA and 10% glycerol). The column was then washed with 10 ml of 0.3 M KCl in Buffer D. The proteins were then eluted from the column using a linear KCl gradient (0.3 M → 0.6 M) in Buffer D. The peak fractions of activity (Fraction IV) were pooled and adjusted to 0.1 M KCl with Buffer D minus KCl. The solution was then applied onto a hydroxylapatite column (1 cm x 5 cm) previously equilibrated with Buffer E (10 mM potassium phosphate, pH 7.5; 1 mM dithiothreitol, and 10% glycerol). The column was then washed with Buffer E containing 0.2 M potassium phosphate buffer, pH 7.5, followed by a linear potassium phosphate gradient (0.2 → 0.5 M) containing 1 mM dithiothreitol and 10% glycerol. The peak fractions of activity were pooled and dialyzed against Buffer D for 2-3 hours to remove phosphate. The dialyzate was immediately concentrated by passage through a small DEAE-cellulose column by one step elution with 0.3 M KCl (Fraction V).

Purification of CO-EIF-1

The 0.1 M KCl wash of the DEAE-cellulose column (step 2) contained most of the CO-EIF-1 activity. Solid ammonium sulfate was added to this 0.1 M KCl wash with stirring to make the final solution 40 percent saturated with ammonium sulfate. The ammonium sulfate suspension was stirred for 15 minutes and then centrifuged at 12,000 xg for 15 minutes. The precipitate was discarded and to the supernatant was added more solid ammonium sulfate to make the final solution 60 percent saturated with ammonium sulfate. The suspension was stirred for 15 minutes and centrifuged at 12,000 xg for 15 minutes. The supernatant was then made 80 percent saturated with ammonium sulfate. After stirring and centrifugation as described above, the final precipitate was suspended in a minimum volume of Buffer D. The suspension was dialyzed overnight against Buffer D with one change.

The dialyzed 60-80 percent ammonium sulfate fractions from 3-4 batches

were pooled and the potassium chloride concentration of the preparation was adjusted to 20 mM with Buffer D minus potassium chloride. The solution was then passed through a DEAE-cellulose column (DE-11, Whatman) (1 cm x 10 cm) previously equilibrated with Buffer D containing 20 mM potassium chloride. The column was then washed with Buffer D containing 50 mM potassium chloride. The CO-EIF-1 activity was eluted from the column using a Buffer D solution containing 0.1 M potassium chloride.

The Met-$tRNA_f^{Met}$ binding to EIF-1 was assayed using a Millipore filtration method as previously described (5).

Other materials and methods were the same as described previously (5).

RESULTS

Table I summarizes the results of the purification of EIF-1 and the effects of the addition of CO-EIF-1 on EIF-1 activity at different stages of purification. The purification procedure was essentially the same as described before (6), except that a phosphocellulose column was run prior to the hydroxylapatite step. The final preparation (Fraction V), when assayed alone, bound 2330 pmole of Met-$tRNA_f^{Met}$ per mg of protein. The addition of CO-EIF-1 to Fractions I and II did not have any significant effect on the EIF-1 activity. Significant stimulation of EIF-1 activity was, however, observed when CO-EIF-1 was added at later stages of purification. The addition of CO-EIF-1 to the Fraction V preparation increased the total Met-$tRNA_f^{Met}$ binding to EIF-1 2.8 fold. Also, the total recovery of the Met-$tRNA_f^{Met}$ binding activity was 2.5 times greater when assayed in the presence of CO-EIF-1; i.e. 30 percent binding was observed in the presence of CO-EIF-1, but only 12 percent in its absence. The specific activity of Fraction V EIF-1 preparation in the presence of CO-EIF-1 was 6,280. This activity corresponds to a binding of input EIF-1 of approximately 90 percent.

As reported previously (6), the purified EIF-1 preparation is extremely unstable. During purification, the EIF-1 activities of the column fractions were assayed immediately after their elution from the column. Aged preparations showed reduced Met-$tRNA_f^{Met}$ binding activities but responded similarly to CO-EIF-1 addition as the fresh preparation.

Fig. 1 describes the Met-$tRNA_f^{Met}$ binding activities at different levels of EIF-1 and CO-EIF-1. Fraction V of the EIF-1 preparation was used in these studies. CO-EIF-1, by itself, did not show any significant Met-$tRNA_f^{Met}$ binding activity. However, the addition of CO-EIF-1 to EIF-1 stimulated significantly the Met-$tRNA_f^{Met}$ binding to EIF-1 at different levels of EIF-1 concentration.

TABLE I

Effects of Addition of CO-EIF-1 to EIF-1 Activity at Different Stages of Purification of EIF-1

Fractions	Total Protein mg	Total Activity Units[a]		Specific Activity Units/mg		Purification fold		Yield %	
		-CO-EIF-1	+CO-EIF-1	-CO-EIF-1	+CO-EIF-1	-CO-EIF-1	+CO-EIF-1	-CO-EIF-1	+CO-EIF-1
Fraction I 0.5 M KCl Ribosomal Wash	1,250	27,000	28,500	22	23	1	1	100	100
Fraction II DEAE-cellulose batch elution, 0-80% $(NH_4)_2SO_4$ concentration	165	18,060	18,200	109	110	5	5	67	64
Fraction III DEAE-cellulose gradient elution	21	7,770	11,100	375	540	17	23	29	39
Fraction IV Phosphocellulose	1.7	3,450	7,900	2,000	4,650	90	202	12	28
Fraction V Hydroxylapatite	1.35	3,140	8,390	2,330	6,280	105	230	12	30

[a] One unit of activity is defined as that amount of protein required to bind 1 pmole of [^{35}S]Met-tRNA$_f^{Met}$ (in the presence of GTP) under the assay conditions.

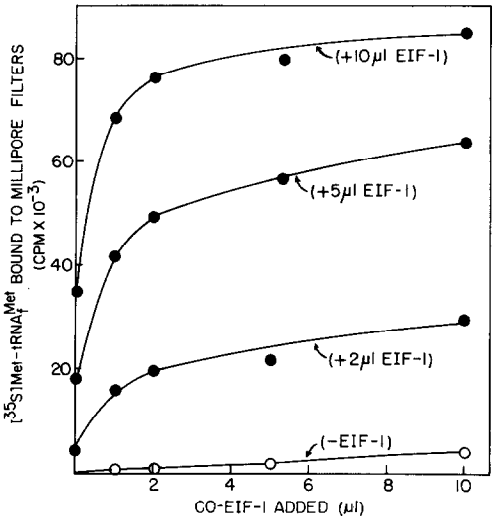

Fig. 1. [^{35}S]Met-tRNA$_f^{Met}$ binding to EIF-1 at varying concentrations of EIF-1 and CO-EIF-1. The standard Millipore filtration assay for [^{35}S]Met-tRNA$_f^{Met}$ binding to EIF-1 was used (5). The incubation mixture contained, in a total volume of 0.075 ml: 20 mM Tris-HCl, pH 7.5; 100 mM KCl; 10 µg bovine serum albumin; 2 mM dithiothreitol; 0.2 mM GTP; 6 pmole [^{35}S]Met-tRNA$_f^{Met}$ (1.5×10^5 cpm); EIF-1 (0.1 mg per ml) and CO-EIF-1 (4 mg per ml) as indicated. The reaction mixture was incubated at 37° for 5 minutes at which time the reaction was terminated by the addition of 3 ml of cold wash buffer (20 mM Tris-HCl, pH 7.5; 100 mM KCl). The solution was then filtered through a Millipore filter. The filter was washed 3 times with 10 ml of cold wash buffer, dried and counted for radioactivity.

Met-tRNA$_f^{Met}$ binding to EIF-1 requires GTP. Fig. 2 shows that the stimulated binding of Met-tRNA$_f^{Met}$ to EIF-1 in the presence of CO-EIF-1 is also clearly GTP dependent. One interesting feature of CO-EIF-1 stimulation of EIF-1 activity is that, under the standard assay conditions, EIF-1 binding to Met-tRNA$_f^{Met}$ shows a significant lag at low EIF-1 concentration, and this lag is overcome in the presence of CO-EIF-1.

Fig. 3 describes the kinetics of Met-tRNA$_f^{Met}$ binding to EIF-1 using Fraction II and Fraction V preparations in the presence and absence of CO-EIF-1. With the Fraction II preparation, the Met-tRNA$_f^{Met}$ binding to EIF-1 is extremely rapid; approximately 80 percent of the maximum Met-tRNA$_f^{Met}$ binding was complete in one minute, and the addition of CO-EIF-1 did not have any effect on the rate of this binding reaction. On the other hand, the rate of Met-tRNA$_f^{Met}$ binding to the Fraction V preparation increased almost linearly for 5 minutes, when a plateau was reached. Approximately 25 percent of the maximum binding was observed after one minute. In the presence of CO-EIF-1, the rate of Met-tRNA$_f^{Met}$

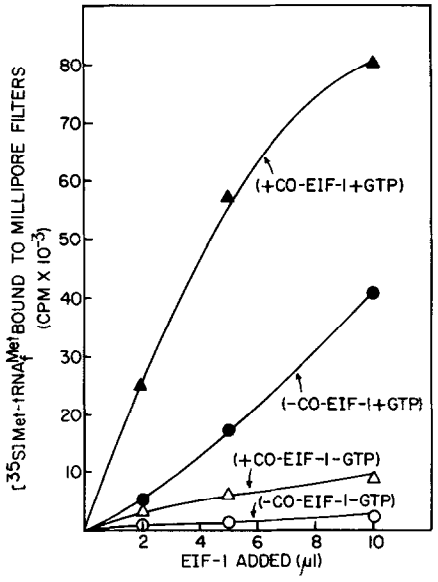

Fig. 2. Requirement of GTP for Met-tRNA$_f^{Met}$ binding to EIF-1 in the presence and absence of CO-EIF-1. Standard Millipore filtration assay conditions as described in Fig. 1 were used. Where indicated 8 µg of CO-EIF-1 was added to the incubation mixture.

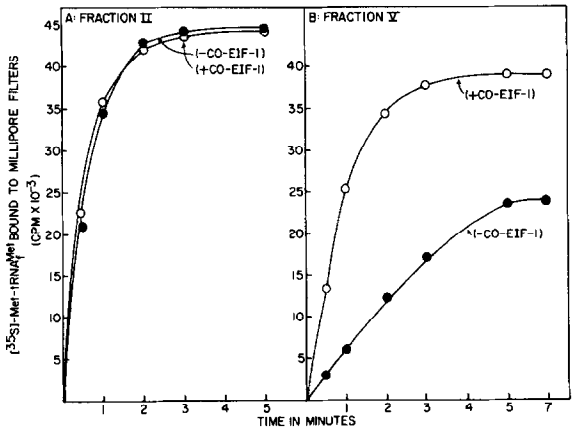

Fig. 3. Kinetics of [^{35}S]Met-tRNA$_f^{Met}$ binding to Fraction II and Fraction V EIF-1 in the presence and absence of CO-EIF-1. Standard Millipore filtration assay conditions were used and the incubation period was varied as indicated in the figure. Concentrations of EIF-1 and CO-EIF-1 used were: EIF-1 Fraction II, 20 µg; EIF-1 Fraction V, 1 µg; CO-EIF-1, 8 µg.

binding to EIF-1 increased significantly; approximately 70 percent of the maximum binding was observed after one minute.

DISCUSSION

The results presented in this paper clearly demonstrate that a protein factor, CO-EIF-1, does not, by itself, bind Met-$tRNA_f^{Met}$, but increases significantly the rate and the extent of Met-$tRNA_f^{Met}$ binding to EIF-1. In the presence of CO-EIF-1, approximately 90 percent of the input EIF-1 (Fraction V) was bound to Met-$tRNA_f^{Met}$. Also, CO-EIF-1 increased significantly the initial rate of Met-$tRNA_f^{Met}$ binding to EIF-1. In the presence of CO-EIF-1, the initial rate of Met-$tRNA_f^{Met}$ binding to purified EIF-1 (Fraction V) approached that of the Fraction II preparation, indicating that in the crude preparation both factors may participate in the binding reaction.

Although the precise mechanism of CO-EIF-1 stimulation of EIF-1 activity is not apparent at present, our preliminary experiments using sucrose density gradient centrifugation suggest that CO-EIF-1 complexes with EIF-1 during Met-$tRNA_f^{Met}$ binding; in the presence of CO-EIF-1, a complex containing Met-$tRNA_f^{Met}$, proteins and GTP sedimented as a higher molecular weight component than the ternary complex, Met-$tRNA_f^{Met}$•EIF-1•GTP. Also, the recovery of the ternary complex during sucrose density gradient centrifugation was significantly greater when CO-EIF-1 was present, indicating that CO-EIF-1 confers stability upon the ternary complex. Several laboratories have suggested (15-16) that EIF-1 activity may be modulated by phosphorylation and there are indications that such phosphorylation may be mediated by a cyclic AMP dependent protein kinase (15). We did not detect any phosphorylation of EIF-1 in the presence of purified CO-EIF-1, ATP and cyclic AMP. Also, the addition of ATP and cyclic AMP did not have any significant effect on EIF-1 binding to Met-$tRNA_f^{Met}$ in the presence or absence of CO-EIF-1.

We have previously reported that EIF-1 can exist in at least two forms; EIF-1A and EIF-1B. The Met-$tRNA_f^{Met}$•EIF-1A•GTP complex formed with highly purified EIF-1 preparations is stable to Mg^{++}, whereas the Met-$tRNA_f^{Met}$•EIF-1B •GTP complex formed with less purified EIF-1 preparations dissociates extensively in the presence of 5 mM Mg^{++}. A protein factor present in the partially purified EIF-1B preparation promotes the Mg^{++} induced dissociation of Met-$tRNA_f^{Met}$•EIF-1B•GTP complex.

Thus, our present results suggest that the Met-$tRNA_f^{Met}$ binding activity is controlled by at least three protein factors; (1) the core enzyme, EIF-1; (2) CO-EIF-1 and (3) the EIF-1A → EIF-1B conversion factor. The precise mechanism by which these protein components bring about the protein synthesis initiation process is presently under investigation.

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