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# Protein synthesis in rabbit reticulocytes: Characteristics of a ribosomal factor that reverses inhibition of protein synthesis in heme-deficient lysates*

(translational regulation/inhibitor of heme-regulated protein synthesis/Met-tRNA$_f^{Met}$-binding factor/eukaryotic initiation factors)

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**ABSTRACT** **A ribosomal salt (0.5 M KCl) wash factor (RF) that reverses inhibition of protein synthesis in heme-deficient reticulocyte lysates has been resolved from the bulk of Met-tRNA$_f^{Met}$-binding factor (EIF-1), Co-EIF-1, and EIF-2 (ternary complex dissociation factor, TDF). The purified RF restores protein synthesis activity of heme-deficient lysates to the level observed in the presence of hemin. No direct correlation exists between amount of EIF-1 activity and ability to reverse inhibition of protein synthesis in heme-deficient lysates. Homogeneous preparations of EIF-1 are completely inactive in reversal of protein synthesis inhibition in heme-deficient lysates. These findings suggest that RF activity is not due to EIF-1 alone but may or may not require EIF-1 as a component of a complex factor.**

Protein synthesis in reticulocyte lysates is regulated by heme (4–6). In heme deficiency, there is rapid activation of a translational inhibitor [HRI, heme-reversible inhibitor, also called HCR (7)] (7–9) that blocks protein chain initiation (5, 6, 8–13). The translational inhibitor HRI has now been extensively purified (14) and has been shown to be a cyclic-AMP-independent protein kinase that specifically phosphorylates the 38,000-dalton subunit of the peptide chain initiation factor EIF-1 (14–18). Recent reports indicate that HRI-phosphorylated EIF-1 may not be recognized by the initiation factors EIF-2 (TDF) (19–20) and also possibly Co-EIF-1 (20, 21), and is inactive in the formation of the initiation complex of Met-tRNA$_f^{Met}$·40S ribosomal subunit (Met-tRNA$_f$·40S) (19–22). An interaction between EIF-1, EIF-2, and Co-EIF-1 is presumably necessary for the formation of 40S initiation complex (1, 23).

Protein synthesis inhibition in heme-deficient lysates can be overcome by addition of the ribosomal salt (0.5 M KCl) wash or a partially purified preparation of EIF-1 (24–27). This observation and the recent reports of possible inactivation of EIF-1 by HRI-induced phosphorylation of the smallest subunit of EIF-1 (19–22) have led to the current belief that HRI inactivates EIF-1 by phosphorylation, thus inhibiting protein synthesis, and addition of exogeneous EIF-1 overcomes this inhibition.

In this paper, we describe the purification of a factor (RF) from the 0.5 M KCl ribosomal salt wash that reverses protein synthesis inhibition in heme-deficient lysates. The RF activity has been resolved from the bulk of EIF-1 activity. Purified RF very efficiently restores protein synthesis in heme-deficient lysates. Equivalent amounts of EIF-1 activity at various stages of purification have little or no ability to reverse protein synthesis inhibition in heme-deficient lysates. Homogeneous EIF-1 preparations have no inhibition reversal activity at any concentration tested.

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## MATERIALS AND METHODS

**Materials.** The materials were obtained from the following sources: [$^{14}$C]leucine (294 Ci/mol) from New England Nuclear; [$^{35}$S]methionine (970 Ci/mmol) from Amersham/Searle; DEAE-cellulose (DE-11 and DE-52) and phosphocellulose (P-11) from Whatman.

**Preparation of Rabbit Reticulocyte Lysates and Protein Synthesis Assay.** Rabbit reticulocyte lysates were prepared according to the method of Hunt *et al.* (9). Preparation of incubation mixtures and measurements of protein synthesis were performed according to the methods of Ranu and London (14). The concentration of exogeneous KCl in the reaction mixture was adjusted to 136 mM unless otherwise stated. The KCl optimum for our lysates is approximately 100 mM. The higher KCl concentration (136 mM) was used in all cases because this is the concentration that exists in the presence of maximum addition of our factors. At 136 mM KCl, protein synthesis in our lysates is inhibited only slightly (less than 10%) during the 40-min incubation period, compared to protein synthesis in the presence of 100 mM KCl. Several different lysates were used during the course of these experiments. These lysates varied in their abilities to incorporate [$^{14}$C]leucine in the absence of hemin. However, the characteristics of the effects of hemin and the various factors on protein synthesis in these lysates were essentially identical. Also, all of the comparisons between hemin and the various factors were made with a single lysate in a single experiment. Protein synthesis was assayed by the incorporation of [$^{14}$C]leucine into protein in 5-$\mu$l aliquots at 30°C; 1.2 nmol of [$^{14}$C]leucine (specific activity 220 cpm/pmol) was added to a standard 25-$\mu$l reaction mixture.

RF activity in fractions from various stages of purification was assayed by its stimulatory effect in a standard 25-$\mu$l reaction mixture without added hemin after 40 min of incubation.

**Assays for Peptide Chain Initiation Factors.** EIF-1 activity was determined by using a Millipore filtration assay for formation of [$^{35}$S]Met-tRNA$_f$·EIF-1·GTP ternary complex as described previously (28). Ternary complex dissociation factor (TDF) activity was determined by using a Millipore filtration assay as described (1). The standard incubation mixture (75 $\mu$l) contained 5–10 pmol of [$^{35}$S]Met-tRNA$_f$ (specific activity 10,000–12,000 cpm/pmol).

**Preparation of EIF-1.** The purification of EIF-1 has been

Abbreviations: The nomenclature for the peptide chain initiation factors is according to Majumdar *et al.* (1). tRNA$_f$, tRNA$_f^{Met}$; EIF-1, Met-tRNA$_f$-binding factor, also called eIF-2 according to Anderson *et al.* (2); EIF-2 (TDF), ternary complex dissociation factor; Co-EIF-1, a factor stimulating Met-tRNA$_f$ binding to EIF-1; HRI, heme-regulated translational inhibitor; 40S (in complexes), 40S ribosomal subunit; RF, the reticulocyte ribosomal salt wash factor that reverses inhibition of protein synthesis in heme-deficient lysates; NaDodSO$_4$, sodium dodecyl sulfate.

* This is paper 23 in a series. Paper 22 is ref. 3.

described (29–31). The homogeneous EIF-1 preparation used in these experiments showed three protein bands upon sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis, corresponding to approximate molecular weights of 54,000, 52,000, and 38,000.

**$NaDodSO_4$ Gel Electrophoresis.** Electrophoresis of proteins in $NaDodSO_4$/polyacrylamide gels was performed according to the method of Laemmli (32). The gels contained 10% acrylamide (10% acrylamide, 0.27% *N,N'*-methylenebisacrylamide). The separating gels were formed as 1.5 × 100 × 150 mm slabs, with 1.5-cm stacking gels (5% acrylamide). Electrophoresis was performed at 150 V for 3 hr in a Bio-Rad model 220 electrophoresis apparatus.

## RESULTS

**Isolation of RF.** The purification procedure up to the second DEAE-cellulose chromatography step is identical with that previously described for the purification of EIF-1 (28). The DEAE-cellulose (DE-52) fractions were scanned for RF activity and EIF-1 activity. The RF and EIF-1 activity profiles are shown in Fig. 1. In the absence of hemin, the lysate used in this experiment showed an incorporation of 30 pmol of [$^{14}$C]leucine. In the presence of hemin (32 μM), 166 pmol of [$^{14}$C]leucine was incorporated. The KCl optimum for the lysate is approximately 100 mM. Determination of [$^{14}$C]leucine incorporation in the presence or absence of hemin in the range of KCl concentrations occurring in the DEAE-cellulose fractions showed a steady decrease in protein synthesis. The RF activity peak shown in Fig. 1 is therefore not due to salt. RF activity is clearly separated from EIF-1 activity and elutes in the TDF region, indicated by the $Mg^{2+}$-induced dissociation of the ternary complex. The RF activity peak, in this experiment, appears rather small because the DEAE-cellulose fractions were dilute and the sensitivity of reticulocyte lysates toward high concentrations of KCl and glycerol prohibits the addition of large amounts of dilute factor. Pooling and concentrating several fractions gave essentially the same profile with higher protein synthesis activity of the RF peak fractions (data not shown).

The peak RF fractions were pooled. The pooled fractions (40 ml, approximately 35 mg of protein) were diluted with buffer A [20 mM Tris·HCl, pH 8.0/1 mM dithiothreitol/50 μM EDTA/10% (vol/vol) glycerol] to adjust the KCl concentration to 0.1 M. The diluted fractions were then loaded on a 1.1 × 7.5 cm DEAE-cellulose column (DE-11) previously equilibrated with buffer A and concentrated by one-step elution with buffer A containing 0.3 M KCl.

The RF activity was further purified by glycerol density gradient centrifugation. Concentrated RF preparation (200 μl, approximately 4 mg/ml) was layered onto a 4.8-ml 14–30% (vol/vol) linear glycerol density gradient containing 20 mM Tris·HCl at pH 8.0, 0.3 M KCl, 1 mM dithiothreitol, and 50 μM EDTA. Twelve gradients were centrifuged in two Spinco SW 50.1 rotors at 45,000 rpm for 11 hr at 2°C. The gradients were then fractionated in an ISCO density gradient fractionator and assayed for RF and EIF-1 activities; 0.3-ml fractions were collected. The results are shown in Fig. 2. In this experiment, 26 pmol of [$^{14}$C]leucine was incorporated in the absence of hemin. The downward trend of the RF activity curve is due to inhibition of protein synthesis by high concentrations of glycerol. Addition of 5 μl of solution containing 30% glycerol (concentration at the bottom of the gradient) lowered [$^{14}$C]-leucine incorporation to approximately 60%. Again, RF activity is clearly separated from the peak of EIF-1 activity. As shown in Fig. 2, one or more minor RF activity peaks were also observed in the glycerol density gradient profile. The significance of these activity peaks is not apparent. These peaks may represent RF complexes with different protein components.

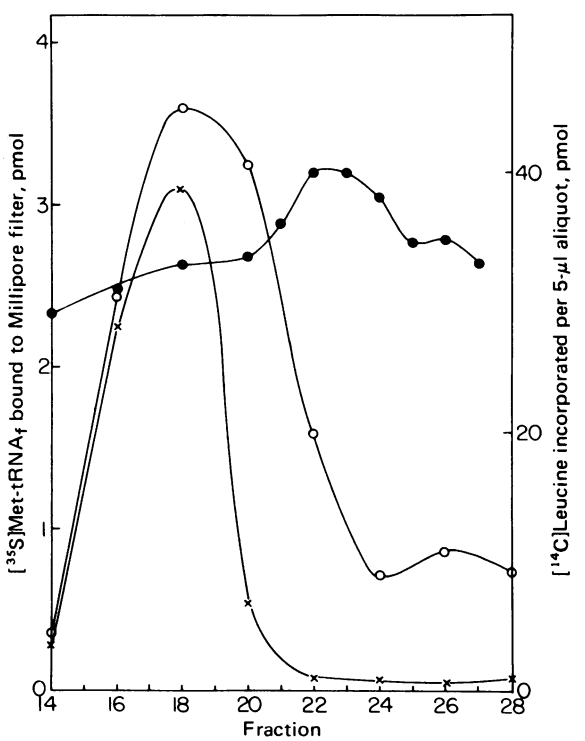


FIG. 1. DEAE-Cellulose chromatography of RF. Chromatography of EIF-1 and RF was performed as described in the text. Fractions contained 6 ml. EIF-1, TDF, and RF were assayed as described under *Materials and Methods*. EIF-1 activity (O) and TDF activity (×) were determined with 15-μl aliquots from column fractions. RF activity (●) was determined with 5-μl aliquots. The range of KCl concentrations present in the reaction mixtures is 109 mM (fraction 14) to 123 mM (fraction 28). The KCl optimum for our lysates is 100 mM.

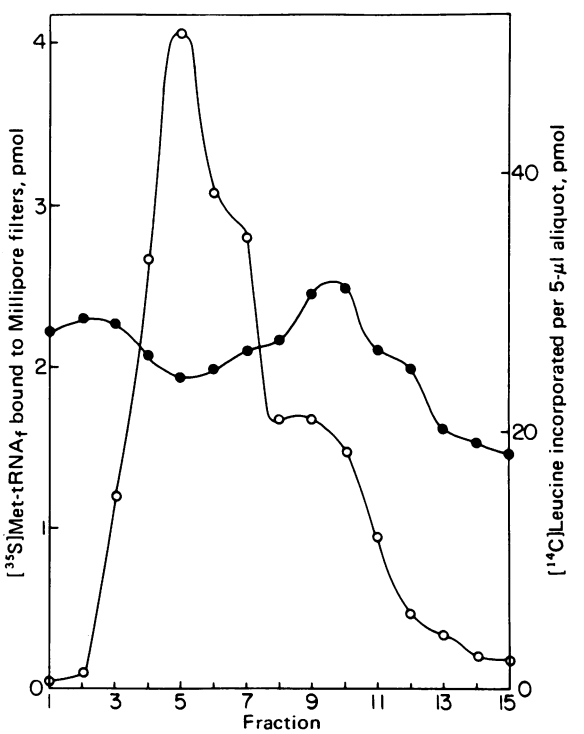


FIG. 2. Glycerol density gradient centrifugation of RF. Density gradient centrifugation of RF was performed as described in the text. EIF-1 activity (O) was determined with 25-μl aliquots from gradient fractions. RF activity (●) was determined with 5-μl aliquots.

The peak fractions containing RF activity were loaded directly onto a 1.1 × 7.5 cm phosphocellulose column (P-11) previously equilibrated with buffer A containing 0.3 M KCl. The column was washed with buffer A containing 0.3 M KCl and was then eluted with a KCl gradient (0.3–0.7 M); 1-ml fractions were collected. The fractions were assayed for RF activity by preparing a master mix without KCl and adjusting the KCl concentration in each assay tube to approximately 120 mM. The peak fractions containing RF activity were diluted to approximately 0.1 M in KCl with buffer A minus KCl, then concentrated by one-step elution from a 1.0 × 5.0 cm DEAE-cellulose column as described above. The purified RF was stored at −4°C.

**Characterization of RF: Comparison of RF and EIF-1.** RF was compared with EIF-1 preparations [fractions IV and V (29–31)] with respect to several known activities (Table 1). Although fraction IV preparations of EIF-1 were equally capable of restoring protein synthesis in inhibited lysates when compared with RF on a microgram basis, the Met-tRNA$_f$-binding activity of the two preparations differed considerably. At comparable concentrations, fraction IV EIF-1 preparation contained 10-fold greater Met-tRNA$_f$-binding activity than RF. Homogeneous EIF-1 preparations (fraction V) showed no RF activity at any concentration tested. The specific activity of fraction V EIF-1 preparation was considerably lower than that of the less purified fraction IV EIF-1 preparation (Table 1). As noted earlier (30), homogeneous EIF-1 preparations (fraction V) are unstable and lose considerable activity upon storage. In the experiments described in Table 1, we have used aged EIF-1 preparations. Similar results were also obtained when freshly prepared fraction V EIF-1 with higher specific activity was used.

Table 1 clearly shows that the ability to restore protein synthesis in heme-deficient lysates is not correlated with amount of EIF-1 activity. The EIF-1 activity in RF preparation was sensitive to incubation at 0°C in the presence of 5 mM $Mg^{2+}$ (TDF activity), whereas the EIF-1 preparations (both fractions IV and V) are essentially free of TDF activity. We have observed that RF activity is occasionally present in partially purified TDF preparations but that RF activity disappears from these preparations during further purification of TDF. Also, because fraction IV EIF-1 preparations containing RF activity do not contain significant amounts of TDF activity, it appears unlikely that the RF activity is due to TDF alone.

**Kinetics of Reversal of Protein Synthesis Inhibition in Heme-Deficient Lysates: Comparison of RF and EIF-1.** The kinetics of restoration of protein synthesis in heme-deficient lysates by RF is shown in Fig. 3*A*. Increasing amounts of RF give increasing reversal of protein synthesis inhibition. Complete reversal of protein synthesis inhibition to the level observed in the presence of hemin has been achieved with fresh preparations of RF. In agreement with previous workers (27), we have observed that RF is capable of reversing protein synthesis inhibition when added after the start of incubation and that RF-stimulated protein synthesis is sensitive to HRI (data not shown).

The kinetics of reversal of protein synthesis inhibition in heme-deficient lysates by comparable amounts of EIF-1 activity in fraction IV and fraction V EIF-1 and RF preparations is shown in Fig. 3*B*. The RF preparation is clearly more active in reversal of protein synthesis inhibition in heme-deficient lysates than fraction IV EIF-1. Fraction V (homogeneous) EIF-1 is completely inactive in reversing protein synthesis inhibition.

**NaDodSO$_4$/Polyacrylamide Gel Electrophoresis of RF, EIF-1, Co-EIF-1, and TDF.** Fig. 4 shows the NaDodSO$_4$/polyacrylamide gel electrophoresis pattern of purified RF, EIF-1, Co-EIF-1, and TDF. As reported previously (31), fraction V EIF-1 preparation gave three protein bands corresponding to approximate molecular weights of 54,000, 52,000, and 38,000 (track 5). These protein bands also precisely coincided with the similar three protein bands of fraction IV EIF-1 preparation (track 4), indicating no detectable damage to any of the subunits of EIF-1. On the other hand, fraction IV EIF-1 preparation, which contains significant RF activity, also shows other protein bands. Again, in agreement with the results of Met-tRNA$_f$-binding experiments, it is clear that RF preparation shows significant but very faint EIF-1 bands in addition to several other protein bands (tracks 1, 2). Some of these protein

Table 1. Comparison of purified RF and EIF-1 (fractions IV and V) preparations for RF, EIF-1, and TDF activities

| Factor | Amount added, μg | RF activity, [$^{14}$C]leucine incorp., pmol | EIF-1 activity, [$^{35}$S]Met-tRNA$_f$ bound, pmol | |
|---|---|---|---|---|
| | | | −$Mg^{2+}$ | +5 mM $Mg^{2+}$ |
| RF | 0.5 | 100.6 | 0.27 | 0.14 |
| | 1.0 | 122.0 | 0.51 | 0.19 |
| | 2.6 | 158.8 | 1.22 | 0.27 |
| Fraction IV EIF-1 | 0.6 | 111.0 | 2.90 | 2.63 |
| | 1.2 | 114.8 | 5.43 | 5.10 |
| | 3.0 | 166.2 | — | — |
| Fraction V EIF-1 | 0.7 | 57.4 | 1.30 | 1.22 |
| | 1.7 | 36.6 | 3.12 | 3.69 |

The controls in the RF activity assay were: −hemin, 68.2 pmol of [$^{14}$C]leucine incorporation; +hemin, 165.0 pmol of [$^{14}$C]leucine incorporation.

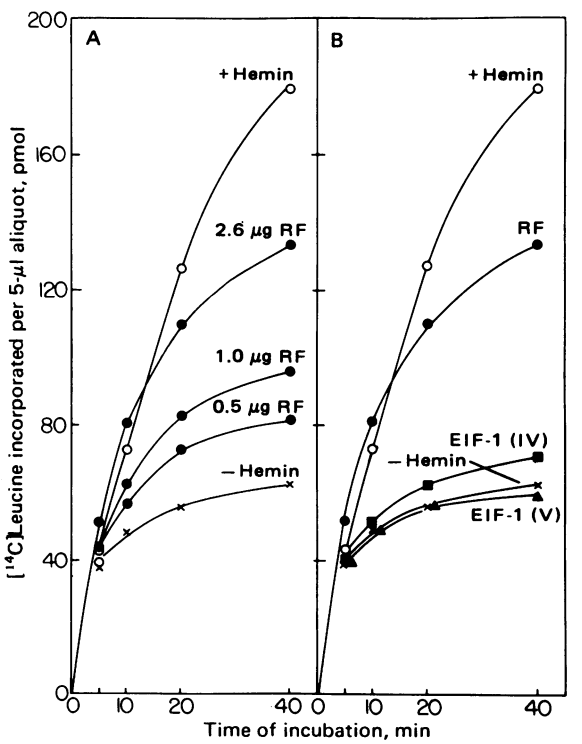


FIG. 3. Kinetics of protein synthesis reversal in heme-deficient lysates: Comparison of RF and EIF-1 activities. (*A*) Reversal of protein synthesis in heme-deficient lysates by increasing levels of RF. Aliquots (5 μl) were taken at the times indicated. (*B*) Comparison of equal amounts of EIF-1 activity (1.2 pmol of [$^{35}$S]Met-tRNA$_f$ bound in the ternary complex formation assay) in RF (2.6 μg) and EIF-1 preparations (fraction IV, 0.3 μg; fraction V, 0.7 μg).

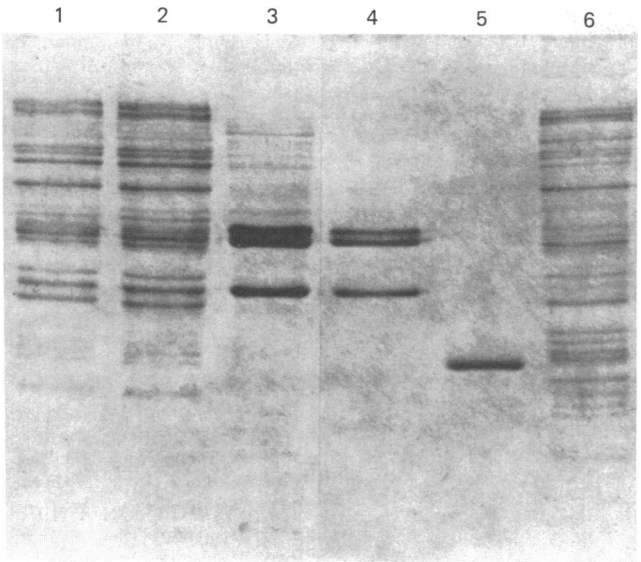


FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis of RF, EIF-1, Co-EIF-1, and TDF. Track 1, 5.0 μg of RF; track 2, 7.5 μg of RF; track 3, 6.0 μg of fraction IV EIF-1; track 4, 4.5 μg of fraction V EIF-1; track 5, 2 μg of Co-EIF-1; track 6, 7.5 μg of TDF. The quantities of fraction IV EIF-1 (track 3) and RF (track 1) have equivalent RF activity.

bands correspond to the protein bands of fraction IV EIF-1 and TDF (track 6), although it has not been possible to identify any specific protein band with RF activity. Homogeneous co-EIF-1 preparation shows a single protein band corresponding to a molecular weight of 20,000. The RF preparation also shows a protein band in this region. These results thus clearly demonstrate that there is no direct correlation between RF activity and EIF-1 activity.

## DISCUSSION

During heme deficiency, a translational inhibitor accumulates in reticulocyte lysates and blocks the initiation step of protein synthesis (5, 6, 8–13). The purified heme-regulated inhibitor, HRI, has been shown to contain a protein kinase activity that specifically phosphorylates the 38,000-dalton subunit of the Met-$tRNA_f$-binding factor EIF-1 (14–18). It is now generally believed that such phosphorylation leads to inactivation of EIF-1 and cessation of protein synthesis. Addition of peptide chain initiation factor preparations containing EIF-1 activity reverses protein synthesis inhibition (24–27), presumably by replacing inactive endogenous EIF-1. Additional indirect support for this hypothesis comes from the observation that, in the presence of HRI and ATP, EIF-1 forms a ternary complex that is not acted upon by initiation factor EIF-2 (19, 20) and is inactive in Met-$tRNA_f$·40S initiation complex formation. It has been postulated that, under the incubation conditions, HRI phosphorylates EIF-1 and such phosphorylated EIF-1 is not recognized by EIF-2 and is thus inactive in peptide chain initiation.

It should be noted, however, that all of the support to date for heme-regulated inhibition of protein synthesis via HRI-catalyzed phosphorylation of EIF-1 comes from indirect evidence. Also, the precise role of EIF-1 in reversal of protein synthesis inhibition in heme-deficient lysates has not been clearly established. For example, it has not yet been possible to demonstrate that isolated phospho-EIF-1 (HRI-catalyzed) is indeed inactive in peptide chain initiation. On the other hand, Trachsel and Staehelin (33) have reported that HRI-phosphorylated EIF-1 participates in several partial reactions of peptide chain initiation and that a peptide chain initiation factor preparation containing phosphorylated EIF-1 is as active in protein synthesis in a reconstituted system as nonphosphorylated EIF-1 (17). Also, under conditions of HRI- and ATP-mediated TDF inhibition, it was observed that, besides EIF-1, three to four polypeptides in TDF are phosphorylated by endogeneous protein kinases in the TDF preparations (20). The roles of such phosphorylated TDF in interaction with EIF-1 (nonphosphorylated or phosphorylated) and in regulation of protein synthesis initiation are not known. Also, there are reports from other laboratories (34–36) which suggest that other factors besides EIF-1 may be involved in reversal of protein synthesis inhibition in heme-deficient lysates. Gross (34) has reported that two initiation factor preparations with different EIF-1 activities could partially reverse protein synthesis inhibition in heme-deficient lysates. Also, Gross (35) and Ranu and London (36) have reported that a postribosomal supernatant factor can reverse protein synthesis inhibition in heme-deficient lysate, although the relationship of this supernatant factor to the ribosomal salt wash factor is not known.

The results presented in this paper clearly demonstrate that homogeneous EIF-1 preparations are completely inactive in reversing protein synthesis inhibition in heme-deficient reticulocyte lysates. The homogeneous preparations of EIF-1 (fraction V) used in these studies showed three protein bands characteristic of pure EIF-1 upon $NaDodSO_4$/polyacrylamide gel electrophoresis, corresponding to molecular weights of approximately 54,000, 52,000, and 38,000. These three protein bands migrated identically with those of partially purified EIF-1 preparations, indicating that no detectable damage to any of the subunits occurred during purification. Moreover, the fraction V preparations were fully active in Met-$tRNA_f$·EIF-1·GTP complex formation, and this ternary complex was active in other partial initiation reactions such as response to Co-EIF-1 (3, 29) and EIF-2 (TDF) (1, 31) as well as Met-$tRNA_f$·40S initiation complex formation.

We have also partially purified a factor (RF) from the 0.5 M KCl ribosomal wash that restores the protein synthesis activity of heme-deficient lysate to the level observed in the presence of optimal concentrations of hemin. We have successfully resolved RF activity from the bulk of EIF-1 activity and have clearly demonstrated that there is no direct correlation between amount of RF activity and amount of EIF-1 activity during different stages of purification. These data demonstrate that the ability to reverse protein synthesis inhibition in heme-deficient reticulocyte lysates (RF activity) is not due to EIF-1 alone. Our RF preparations still contain significant amounts of EIF-1 activity, as well as other peptide chain initiation factor activities such as EIF-2 (TDF). The possibility, therefore, remains open that RF activity may either be due to EIF-1 in association with other components or occur by a mechanism entirely independent of EIF-1 activity. The plausible mechanisms of RF action may include: (i) inactivation of HRI and (ii) reactivation of EIF-1 or other initiation factors rendered inactive by HRI.

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