Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 6998–7002, November 1984
Biochemistry

Distribution of reversing factor in reticulocyte lysates during active protein synthesis and on inhibition by heme deprivation or double-stranded RNA

(phosphorylation of eukaryotic initiation factor 2 α subunit/regulation of protein synthesis/inhibition of protein chain initiation/reversing factor·eukaryotic initiation factor complexes)

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Contributed by Irving M. London, July 26, 1984

ABSTRACT **We have recently shown a direct correlation between protein synthetic activity and the function of reversing factor (RF) as a catalyst of GDP–GTP exchange in whole reticulocyte lysates under normal conditions and on inhibition of protein synthesis by heme deficiency, double-stranded RNA, or oxidized glutathione. In this paper we report that RF is detectable as a nonribosomal complex with eukaryotic initiation factor 2 phosphorylated in its α subunit [eIF-2(αP)] in whole lysates inhibited by heme deprivation or by double-stranded RNA. The complex contains no unphosphorylated eIF-2α, and the GDP present is freely dissociable. All nonribosomal eIF-2(αP) is complexed with RF in fully inhibited lysates; we have not detected free eIF-2(αP). RF in this [RF·eIF-2(αP)] complex is unavailable to catalyze the release of GDP from eIF-2·GDP. Dephosphorylation of eIF-2(αP) present in nonribosomal fractions releases active RF, which is able to carry out its normal guanine nucleotide exchange function.**

During the initiation of protein synthesis, the 60S ribosomal subunit joins with the 48S preinitiation complex to form the 80S initiation complex. This process is believed to be accompanied by the hydrolysis of eukaryotic initiation factor 2 (eIF-2) bound GTP to GDP (1–4). In order to allow this eIF-2 to take part in a further round of initiation, the GDP moiety must be replaced by GTP, whose affinity for eIF-2 is much weaker than that of GDP (5, 6). A multipolypeptide factor has been isolated, here designated reversing factor (RF), that appears to catalyze this exchange, thereby stimulating ternary complex formation [eIF-2·Met-tRNA$_f$·GTP] and ensuing reinitiation (7–11).

The inhibition of protein synthesis caused by heme deprivation or by double-stranded RNA (ds RNA) is accompanied by the activation of specific protein kinases that phosphorylate the α subunit of eIF-2 (eIF-2α) (for reviews, see refs. 12–14); these kinases, respectively, are heme-regulated eIF-2α kinase (HRI) and ds RNA-regulated eIF-2α kinase (dsI). However, protein synthesis is inhibited in lysates when only 30–40% of the eIF-2α present is phosphorylated [eIF-2(αP)] (15, 16), an observation that is explainable if a factor other than eIF-2 is limiting. Results of experiments *in vitro* indicate that RF reacts with the phosphorylated binary complex eIF-2(αP)·GDP to produce a nonfunctional RF·eIF-2(αP) complex, which is incapable of stimulating the dissociation of GDP from unphosphorylated eIF-2 (8, 17). Since there is only approximately 5–10% as much RF in reticulocyte lysates as there is eIF-2 (our unpublished observations, based on yields of purified factors), phosphorylation of a small proportion of the total eIF-2α is sufficient to sequester RF, inhibiting the recycling of eIF-2 and the initiation of protein synthesis.

We have shown that the kinetics of loss of RF function are closely correlated with the inhibition of protein synthesis in whole reticulocyte lysates (18). We report here that, in whole lysates inhibited by heme deprivation or ds RNA, RF is sequestered as a nonribosomal complex with eIF-2(αP). Dephosphorylation of eIF-2(αP) by treatment of nonribosomal fractions with alkaline phosphatase liberates functional RF, which is able to catalyze the release of GDP from eIF-2·GDP. No unphosphorylated eIF-2 is observed in the complex, and GDP present is freely exchangeable with exogenous GDP. Further, all nonribosomal eIF-2(αP) is present exclusively as a complex with RF. We propose that RF binds to ribosomal eIF-2(αP)·GDP, releases the complex from the ribosome, and then is sequestered as a nonribosomal 15S complex RF·eIF-2(αP). The data extend our findings *in situ* (18) and demonstrate that the RF·eIF-2(αP) complex observed *in vitro* (8, 17) is present in the whole lysate. These findings provide further evidence that RF is sequestered in this complex, leading to the inhibition of protein synthesis.

MATERIALS AND METHODS

Preparation of RF. The reversing factor was isolated from reticulocyte lysates as described (8).

Preparation of HRI. The heme-regulated inhibitor was isolated from postribosomal supernatant by standard procedures (19).

Assay of RF. RF activity was measured by its ability to stimulate the dissociation of labeled GDP from the binary complex eIF-2·[^{3}H]GDP as described (8). The eIF-2·[^{3}H]GDP complex was preformed by incubating 1.5–2 pmol of eIF-2 (80% pure)/2 μM [^{3}H]GDP (6500 cpm/pmol)/20 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/100 mM KCl with creatine kinase as carrier (100 μg/ml) for 10 min at 30°C. The reaction mix was kept on ice and stabilized by the addition of 1 mM $Mg(OAc)_2$ prior to use. eIF-2·[^{3}H]GDP was added to a gradient fraction previously supplemented to contain 100 μg of creatine kinase per ml and 40 μM GDP (20,000-fold excess over the added [^{3}H]GDP). After 12 min at 30°C, the eIF-2·[^{3}H]GDP remaining undissociated was determined by binding to a Millipore membrane, as previously described (18).

Protein Synthesis. Protein synthesis mix was prepared ac-

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Abbreviations: RF, reversing factor (also referred to in the literature as anti-HRI, eRF, sRF, GEF, and eIF-2B); eIF-2, eukaryotic initiation factor 2; eIF-2α, α subunit (M_r 38,000) of eIF-2; eIF-2(αP), eIF-2 phosphorylated in its α subunit; HRI, heme-regulated eIF-2α kinase; ds RNA, double-stranded RNA; dsI, ds RNA-regulated eIF-2α kinase.

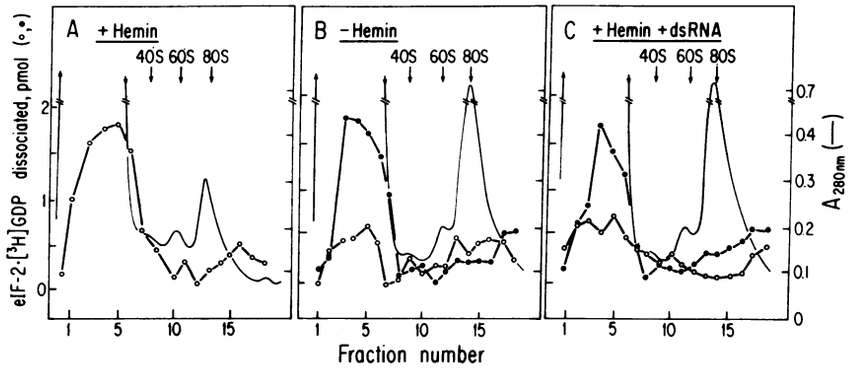

FIG. 1. Localization of RF in whole lysates. Standard reticulocyte-lysate protein-synthesis mixes (250 μl total) were incubated for 15 min at 30°C with 20 μM hemin Cl (*A*), without hemin Cl (*B*), and with 20 μM hemin Cl and 15 ng of reoviral ds RNA per ml (*C*). These samples were cooled on ice, diluted with buffer containing 25 mM KF to inhibit dephosphorylation of eIF-2(α*P*) (21) and immediately were fractionated on sucrose gradients containing no KF. Fractions were either assayed for RF activity after treatment with 2.2 units of bovine intestinal alkaline phosphatase per 400-μl fraction for 30 min at 30°C to dephosphorylate proteins, including eIF-2(α*P*) (●) or after incubation in the absence of alkaline phosphatase (○). The RF assays contained 1.5–2 mol of eIF-2·[^{3}H]GDP. Alkaline phosphatase does not affect the assay.

cording to Pelham and Jackson (20) but without nuclease treatment of the reticulocyte lysate.

Sucrose Gradient Fractionation. After incubation, protein synthesis mixes (typically 250 μl) were chilled on ice and were diluted with 2 vol of ice-cold 1.5× TKMF buffer (1× TKMF buffer is 25 mM Tris·HCl, pH 7.6/25 mM KCl/2 mM $Mg(OAc)_2$/25 mM KF) and layered over 12 ml of exponential 15.89–27.81% sucrose gradients in the same buffer or in buffer containing no KF. Centrifugation was at 196,000 × g_{avg} at 2°C for 3 hr in a Beckman SW 41 Ti rotor. Gradients were collected by upward displacement with continuous monitoring at 280 nm in an ISCO fractionator.

Glycerol Gradient Fractionation. Protein synthesis mixes (typically 125 μl) were chilled and diluted as above and layered on 5 ml of 15–50% linear glycerol gradients in TKMF buffer. Tubes were centrifuged at 200,000 × g_{avg} at 0°C for 18 hr in a Beckman SW 50.1 rotor. Gradients were collected as described above.

Quantitation and Characterization of Protein-Bound Guanine Nucleotide. Protein-bound ^{32}P-labeled guanine nucleotide was quantitated by determination of Cerenkov radiation bound to undried Millipore membranes (HAWP 02500). Counting efficiency was the same as for dried membranes. The bound nucleotide was then eluted from each filter by extraction with 2 0.5-ml vol of ice-cold 5% trichloroacetic acid. The trichloroacetic acid was removed by extraction with 7 1-ml vol of ether. The extracts were concentrated by lyophilization and redissolved in 10 μl of deionized water. The concentrated extracts (5 μl) were spotted on polyethyleneimine-cellulose plates, and the nucleotides were separated by ascending chromatography with 1 M potassium phosphate buffer (pH 3.4). Nucleotides were visualized by autoradiography and identified by reference to known standards.

Materials. GDP, ATP, and creatine kinase were purchased from Sigma; bovine intestinal alkaline phosphatase, from Boeringer Mannheim; and [8-^{3}H]GDP (9 Ci/mmol; 1 Ci = 37 GBq), [α-^{32}P]GTP (3000 Ci/mmol), [^{32}P]orthophosphate (50 mCi/ml), and [γ-^{32}P]ATP (1000–3000 Ci/mmol), from New England Nuclear. Factor eIF-2 and reovirus ds RNA were generously provided by Daniel Levin (Massachusetts Institute of Technology).

RESULTS AND DISCUSSION

Localization of RF. The presence of RF in initiation complexes was determined under physiological conditions in the reticulocyte-lysate cell-free system. Lysates were incubated as described and analyzed immediately on sucrose gradients. RF was localized in gradient fractions by the functional GDP-exchange assay (18)—*i.e.*, by its ability to stimulate the dissociation of labeled GDP from the binary complex eIF-2·[^{3}H]GDP. Under conditions that lead to the phosphorylation of eIF-2α, fractions were first treated with alkaline phosphatase to dephosphorylate eIF-2(α*P*) in nonfunctional

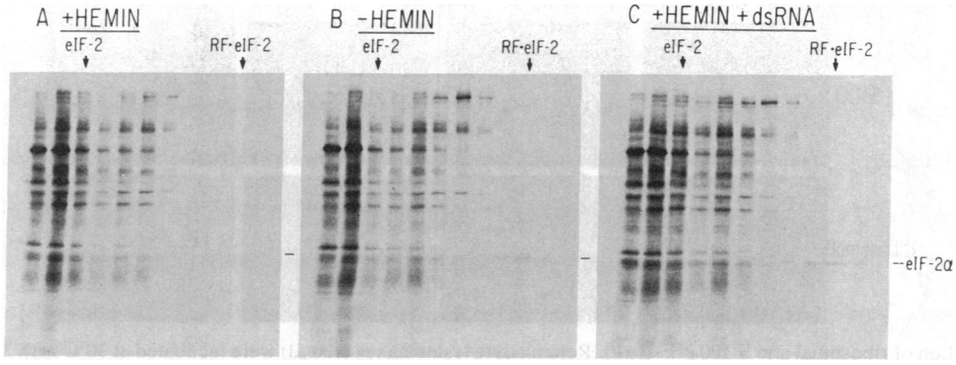

FIG. 2. Phosphorylation state of nonribosomal eIF-2α in the RF·eIF-2 complex. Reticulocyte-lysate cell-free protein-synthesis mixes (125 μl total) were incubated as described in the legend to Fig. 1 (*A–C*) except that [γ-^{32}P]ATP (100 μCi) was added at 10 min. Samples taken at 15 min were immediately fractionated on glycerol gradients containing KF. Each gradient fraction was adjusted to pH 5 by the addition of 1 M HOAc, and protein was precipitated on ice for 1 hr. Precipitates were collected by centrifugation and pellets were resuspended in protein dissociation buffer and electrophoresed in a 0.1% $NaDodSO_4$/15% acrylamide/0.086% bisacrylamide gel (22). The position of purified, phosphorylated eIF-2α on the gel in the autoradiogram is noted.

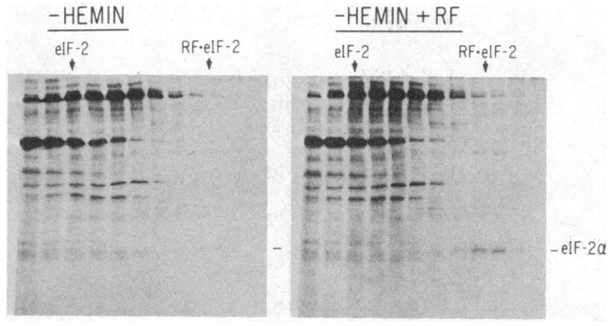

FIG. 3. Further characterization of the RF·eIF-2(αP) complex: effect of adding purified RF. Incubations were carried out and the samples were processed as described for Fig. 2. (A) Heme-deficient lysate. (B) Heme-deficient lysate with purified RF (about 25 pmol) added at 10 min.

RF·eIF-2(αP) complexes, with resultant release of the RF to take part in the GDP-exchange assay. The results are shown in Fig. 1.

RF is present under all conditions predominantly as a nonribosomal complex. In heme-deficient lysate (Fig. 1*B*) or in lysate inhibited by ds RNA (Fig. 1*C*), the RF is present in a nonfunctional form but is activated by incubation with alkaline phosphatase. Treatment with alkaline phosphatase leads to the dephosphorylation of many phosphoproteins, including 80–90% of the eIF-2(αP) in the gradient (data not shown). The results of these experiments are consistent with the view that RF forms a nonribosomal complex with eIF-2(αP), since we have shown previously (8) that RF in the RF·eIF-2(αP) complex is unable to chase GDP from eIF-2·GDP.

Identification of eIF-2(αP) as a Complex with RF. Reticulocyte lysates were incubated with [γ-^{32}P]ATP, and nonribosomal complexes were fractionated by sedimentation through glycerol gradients. ^{32}P-phosphorylated eIF-2 and RF·eIF-2 were prepared *in vitro* from purified components (8) and centrifuged in gradients run in parallel. Their sedimentation positions are noted. The phosphoprotein profiles are shown in Fig. 2. At a time when protein synthesis has ceased in a heme-deficient lysate or in a lysate incubated with ds RNA, eIF-2(αP) sediments at exactly the same position as does purified RF·eIF-2 (Fig. 2 B and C, respectively). In addition, the amount of this eIF-2α-containing complex increases when purified RF is added to the heme-deficient lysate (compare Fig. 3*B* with Fig. 3*A*). These results strongly suggest that both RF and eIF-2(αP) form part of the same nonribosomal complex in inhibited lysates. Further, the complex cannot contain significant amounts of proteins other than RF and eIF-2 because this would have severely altered the sedimentation characteristics. We have been unable to detect nonribosomal eIF-2(αP) free of RF in these studies; therefore, all nonribosomal eIF-2(αP) is present exclusively as the complex with RF. This accounts for 5–10% of the total eIF-2(αP), the remainder being associated with the ribosomal fraction (Fig. 4). A similar distribution of phosphorylated eIF-2α between the S100 and ribosomes has been reported in Ehrlich ascites cells (21).

RF Is Not Complexed with Unphosphorylated eIF-2α in Inhibited Lysate. We have shown in *in vitro* studies (8) that RF is inactivated when complexed with eIF-2(αP) but not with unphosphorylated eIF-2α. Thus, in order to sequester RF, only eIF-2(αP) should be present in the RF·eIF-2 complex in fully-inhibited lysates. To test this, heme-replete and heme-deficient lysates were incubated without label for 15 min, and their nonribosomal complexes were separated on glycerol gradients. Each gradient fraction was subsequently incubated with [γ-^{32}P]ATP and purified HRI to phosphorylate any unphosphorylated eIF-2α present. GDP (5 μM) was also added because HRI will not efficiently phosphorylate eIF-2α in a RF·eIF-2 complex in the absence of GDP (8). The results (Fig. 5) show that, while there is unphosphorylated eIF-2α in the RF·eIF-2 complex in a heme-replete lysate, there is no detectable unphosphorylated eIF-2α in a similar complex in a heme-deficient lysate.

GDP in the Sequestered RF·eIF-2(αP) Complex Is Freely Dissociable. To study further the mechanism by which the nonfunctional RF·eIF-2(αP) complex is formed, we examined the effect of the prephosphorylation of the binary complex on the kinetics of RF-stimulated GDP dissociation *in vitro*. RF catalytically stimulated the complete exchange of [^{3}H]GDP from unphosphorylated eIF-2·[^{3}H]GDP complexes at a rate dependent upon the concentration of RF present (Fig. 6). However, if the binary complex is phosphorylated prior to the addition of RF, there is only a limited, rapid dissociation of [^{3}H]GDP from the complex. The extent of [^{3}H]GDP exchanged from the phosphorylated binary complex was proportional to the amount of RF present in the assay, with a stoichiometry of 1 pmol of [^{3}H]GDP dissociated per pmol of RF added. The data indicate that the mechanism by which RF becomes inactivated by eIF-2α phosphorylation involves the direct binding of RF to the phosphorylated binary complex [eIF-2(αP)·GDP] from which the GDP becomes dissociable; the RF·eIF-2(αP) complex formed is nondissociable and therefore nonfunctional.

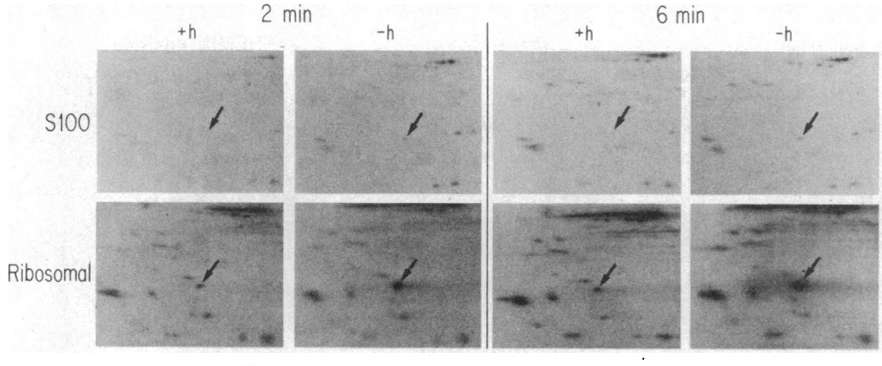

FIG. 4. Quantitation of ribosomal and S-100 eIF-2(αP). Reticulocyte lysate mixes (100 μl) were incubated at 30°C with 20 μM hemin (+h) or without (−h) for 2 and 6 min. After the addition of 50 mM NaF/5 mM EDTA on ice to inhibit further kinase/phosphatase activity, samples were layered over 0.5-ml cushions of 20% (vol/vol) glycerol/50 mM NaF/5 mM EDTA/10 mM Tris·HCl, pH 7.7/50 mM KCl, and the ribosomes were pelleted by centrifugation at 130,000 × g_{avg} at 4°C for 90 min in a Beckman Ti 50 rotor. The EDTA does not cause significant dissociation of eIF-2(αP) from ribosome-bound complexes (unpublished observation). Proteins in the supernatant (S-100) were precipitated at pH 5.2 and resuspended in sample buffer (15) before electrophoresis. The ribosome pellet was resuspended in sample buffer and analyzed directly. The position of eIF-2(αP) is indicated by the arrow. Labeling of lysate proteins with $^{32}P_i$ (150 μCi), two-dimensional gel electrophoresis, and autoradiography were as described (15).

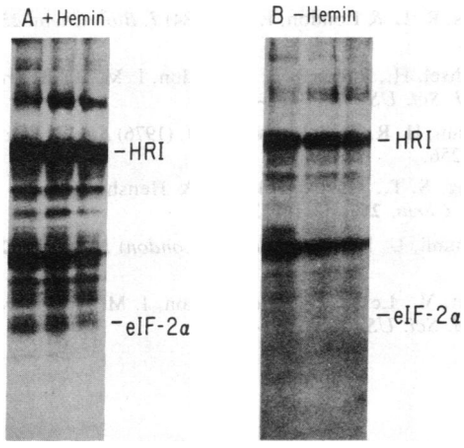

FIG. 5. Phosphorylation state of eIF-2α in the RF·eIF-2 complex. Reticulocyte lysate mixes (125 μl) were incubated for 15 min at 30°C with 20 μM hemin Cl (*A*) or without 20 μM hemin Cl (*B*) and immediately fractionated on glycerol gradients containing KF. Unphosphorylated eIF-2α in one quarter (60 μl) of each gradient fraction was phosphorylated *in vitro* with 0.05 μg of purified HRI, 10 μCi of [γ-^{32}P]ATP, 20 μM ATP, 20 μM $Mg(OAc)_2$, and 5 μM GDP for 10 min at 30°C. Reactions were terminated by the addition of protein dissociation buffer, and electrophoresis was as described for Fig. 2. The figure is an autoradiogram.

To investigate the presence of GTP and GDP in the RF·eIF-2(αP) complex under physiological conditions, incubations were carried out as described for Fig. 2 except that instead of [γ-^{32}P]ATP, [α-^{32}P]GTP was added at the beginning of the incubation. The samples were fractionated on glycerol gradients, and the amount of protein-bound guanine nucleotide in each fraction was determined by assaying ^{32}P radioactivity bound to Millipore filters. A minor peak of guanine nucleotide, determined to be GDP as described in *Methods*, was observed to comigrate with the RF·eIF-2(αP) complex in a heme-deficient lysate supplemented with RF. However, the GDP in this RF·eIF-2(αP)·GDP complex was readily exchanged with free unlabeled GDP added to each fraction before binding to Millipore filter (data not shown). Glycerol gradient centrifugation of the RF·eIF-2(αP) complex formed *in vitro* upon binding purified RF to the phosphorylated binary complex containing [^{3}H]GDP [eIF-2(αP)·[^{3}H]GDP] confirmed that no [^{3}H]GDP remains tightly bound to eIF-2 upon the formation of the RF·eIF-2(αP) complex.

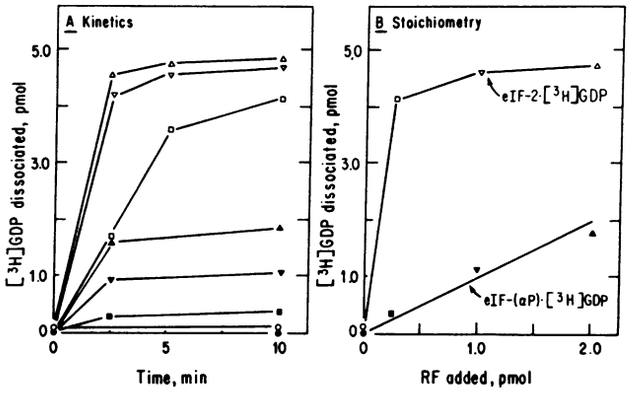

FIG. 6. Effect of eIF-2α phosphorylation on the kinetics and stoichiometry of RF-catalyzed dissociation of GDP. The eIF-2·[^{3}H]GDP complex was formed by incubating (10 min, 30°C) assay mixtures containing 20 mM Tris·HCl (pH 7.6), 100 mM KCl, 0.5 mM dithiothreitol, creatine kinase at 100 μg/ml as carrier, 50 μM ATP, 2 μM [^{3}H]GDP (6500 cpm/pmol), and 5 pmol of eIF-2. The eIF-2·[^{3}H]GDP complex was stabilized by the addition of $Mg(OAc)_2$ to 1 mM, and the complex was incubated for an additional 10 min with or without the addition of 0.5 μg of HRI. Excess ATP was removed by the addition of 0.25 unit of hexokinase and 5 mM glucose for 5 min. The ability of RF to catalyze GDP exchange was subsequently measured upon the addition of 40 μM unlabeled GDP and RF by the Millipore filter binding method as described (8). (*A*) Rate of dissociation of GDP from eIF-2·[^{3}H]GDP (○, □, ▽, △) and eIF-2(αP)·[^{3}H]-GDP (●, ■, ▼, ▲) complexes in the absence of RF (○, ●) and upon addition of 0.25 pmol of RF (□, ■), 1.0 pmol of RF (▽, ▼), and 2.0 pmol of RF (△, ▲). (*B*) A plot of the pmol of [^{3}H]GDP dissociated in 10 min from unphosphorylated binary complex (○, □, ▽, △) and phosphorylated binary complex (●, ■, ▼, ▲) against the amount of RF added.

CONCLUSION

The results presented here show that RF is sequestered predominantly as a nonribosomal complex in lysates inhibited by heme deprivation or by ds RNA (Fig. 1). However, the data do not preclude transient binding of RF to ribosomal complexes. The complex contains only eIF-2(αP) (Figs. 2 and 5*B*) and contains freely dissociable guanine nucleotide (Fig. 6). Since no free eIF-2(αP) is present in nonribosomal fractions, we suggest that the ribosome-associated eIF-2(αP)·GDP complex may be released from the ribosome upon interaction with RF. Although it is not the only possible explanation, this model would account simply for the fact that the only nonribosomal eIF-2(αP) is present as a stoichiometric complex with RF.

Dephosphorylation by a phosphatase is required to liberate functional RF from the complex (Fig. 1 *B* and *C*). Further, since a concomitant dephosphorylation of eIF-2(αP) is observed when protein synthesis recommences upon addition of hemin to a heme-deficient lysate (23), we propose that phosphorylated eIF-2(αP) in the RF·eIF-2(αP) complex is the normal substrate for the physiological phosphatase that acts to dephosphorylate eIF-2(αP), liberating RF with consequent restoration of protein synthesis.

It should be emphasized that in the absence of functional RF, GDP is not dissociable in the eIF-2·GDP complex and, accordingly, the formation of the ternary complex eIF-2·GTP·Met-tRNA$_f$ is inhibited. When RF is sequestered in the RF·eIF-2(αP)·GDP complex, the RF is nevertheless able to promote the dissociation of GDP from this complex; it is, however, unavailable for the dissociation of eIF-2·GDP generally.

This work was supported by U.S. Public Health Service Grant AM-16272. N.S.B.T. was supported by a Damon Runyon–Walter Winchell Cancer Fund Fellowship (DRG-697), and R.L.M. was supported by a fellowship from the National Foundation for Cancer Research.

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