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Effect of phosphorylation of the α-subunit of eukaryotic initiation factor 2 on the function of reversing factor in the initiation of protein synthesis

(regulation of protein synthesis/inhibition of protein chain initiation/binary and ternary initiation complexes)

ROBERT L. MATTS, DANIEL H. LEVIN, AND IRVING M. LONDON

Harvard University-Massachusetts Institute of Technology Division of Health Sciences and Technology and the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT **The reticulocyte reversing factor (RF) isolated as a complex with eukaryotic initiation factor 2 (eIF-2) acts catalytically in restoring protein synthesis in reticulocyte lysates inhibited by heme deficiency. In reconstituted *in vitro* assay mixtures containing Mg^{2+} (0.25–0.5 mM), RF catalyzes the formation of the binary complex (eIF-2·GDP) but this effect is inhibited when eIF-2 is phosphorylated by the heme-regulated kinase for the α-subunit of eIF-2 (HRI). More significantly, RF catalyzes the rapid dissociation of (eIF-2·GDP), which permits the exchange of GTP for GDP and, in the presence of Met-tRNA$_f$, promotes the formation of the ternary complex (eIF-2·Met-tRNA$_f$·GTP). However, phosphorylation of the binary complex by HRI prevents its dissociation by RF and, as a consequence, ternary complex formation is inhibited. Our results indicate that phosphorylated binary complex [eIF-2(αP)·GDP] interacts with RF to form a [RF·eIF-2(αP)] that is not readily dissociable. This binding of RF renders it unavailable to catalyze the dissociation of unphosphorylated binary complex, thereby blocking the recycling of eIF-2. Since RF is present in lysates at a limited concentration relative to that of eIF-2, the sequestering of RF in this manner could account for the observation that the phosphorylation of a small proportion of eIF-2 in heme-deficient lysates is sufficient to inhibit protein synthesis.**

The inhibition of initiation of protein synthesis in heme-deficient rabbit reticulocytes and their lysates is due primarily to the activation of a cAMP-independent heme-regulated protein kinase (HRI) that specifically phosphorylates the 38-kilodalton (kDa) α-subunit of the eukaryotic initiation factor eIF-2 (eIF-2α) (1–5). Restoration of linear synthesis in inhibited lysates can be achieved by the addition of hemin (20 μM) (6) or of high (nonphysiological) levels of eIF-2 (7, 8). Recently, a multipolypeptide factor that can prevent or reverse inhibition of protein synthesis in heme-deficient or HRI-inhibited lysates has been described by several groups. Variously designated anti-HRI (9), RF (10, 11), or sRF (12), the factor has been purified from both post-ribosomal supernatants and ribosomal salt washes in either a free form or complexed in a 1:1 stoichiometry with eIF-2 (9, 10). We shall refer to it as RF or RF·eIF-2. Earlier studies with similar factors called ESP (13), SF (14), and co-eIF-2C (15) suggested that they stimulated ternary complex (eIF-2·Met-tRNA$_f$·GTP) formation by interacting with eIF-2 and that this interaction is inhibited by phosphorylation of eIF-2α. Subsequent studies in which a complex of RF and eIF-2 was used supported these findings and suggested a role for RF in the recycling of eIF-2 by a mechanism that is blocked by the phosphorylation of eIF-2α (16, 17).

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This mechanism is now partly understood. In the initiation cycle, the joining of the 48S complex and the 60S ribosomal subunits is accompanied by the hydrolysis of GTP to GDP and the release of eIF-2 (18, 19), presumably as a binary complex (eIF-2·GDP). To recycle eIF-2 and permit formation of the ternary complex (eIF-2·Met-tRNA$_f$·GTP), the GDP moiety in the binary complex must be replaced by GTP, whose affinity for eIF-2 is much weaker than that of GDP (20, 21). RF appears to catalyze this exchange, thereby stimulating ternary complex formation when Met-tRNA$_f$ is also present (16). Phosphorylation of eIF-2α interferes with this mechanism (16, 22) by preventing the exchange of GTP for GDP bound to eIF-2. These findings are consistent with a role for RF in the recycling of eIF-2. In this paper, we report results that suggest that RF can react with phosphorylated binary complex to form a [RF·eIF-2(αP)] complex that is nonfunctional.

METHODS AND MATERIALS

Preparation of RF. The reversing factor was isolated from reticulocyte lysates by a modification of the procedure described by Siekierka *et al.* (10). Step 1: lysate S100 (250 ml) was applied to a Sepharose 6B-heparin column (2.5 × 21 cm) (23) that had been washed with buffer A (20 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/0.2 mM EDTA/10% glycerol)/100 mM KCl. RF activity was eluted with buffer A/500 mM KCl. Step 2: after dialysis, RF was applied to a DEAE-cellulose column (1.5 × 10 cm), washed as above, and eluted with buffer A/250 mM KCl. Step 3: after dialysis against buffer A/50 mM KCl, RF was applied to a CM-Sephadex A-50 column (0.75 × 10 cm) and eluted with buffer A/200 mM KCl. Step 4: eluant RF was applied to a Sepharose 6B-heparin column (0.75 × 17.5 cm) and eluted at 300–400 mM KCl. Step 5: after dialysis and concentration, RF aliquots (0.175 ml, 30 μg) were centrifuged in 4.8-ml linear 15–50% glycerol gradients in buffer A (plus 100 mM KCl) at 45,000 rpm (2°C) for 18 hr in a Beckman SW 50.1 rotor. RF fractions were stored at −70°C. This RF preparation (containing five polypeptide subunits) was isolated as a complex with eIF-2 (9, 10) (Fig. 1). The glycerol gradient RF (step 5) was used in all of the experiments described here and is referred to as RF or as RF·eIF-2.

eIF-2 (80% pure) free of RF (after glycerol gradient centrifugation) was prepared by standard procedures from a reticulocyte ribosomal salt wash by one of us (D.H.L.).

Abbreviations: eIF-2, eukaryotic initiation factor 2; eIF-2α, α-subunit (38 kilodaltons) of eIF-2; HRI, heme-regulated eIF-2α kinase; kDa, kilodalton(s); RF or RF·eIF-2, reversing factor; (eIF-2·GDP), binary complex containing eIF-2; [RF·eIF-2(αP)], RF·eIF-2 phosphorylated in eIF-2α.

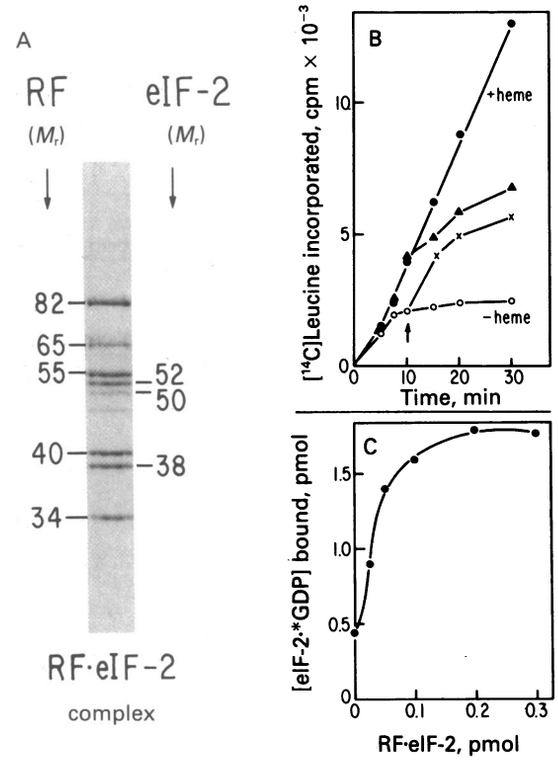

FIG. 1. Two assays for RF: (*i*) Effect on protein synthesis in heme-deficient lysates and (*ii*) stimulation of binary complex (eIF-2·[^{3}H]GDP) formation. (*A*) The peak fraction of RF from the glycerol gradient step was used in all assays; 2 μg of this preparation was analyzed by electrophoresis 0.1% NaDodSO$_4$/10% acrylamide/0.26% bisacrylamide (1) and the gel was stained with Coomassie blue. Except for the β-subunit (50 kDa) of eIF-2, the five subunits of RF and the α (38 kDa)- and γ (52 kDa)-subunits of eIF-2 appear to be equivalent. (*B*) Protein synthesis assay. Standard lysate-protein-synthesis assays (30 μl) were carried out for 30 min at 30°C as described (6, 24). ●, With 20 μM hemin Cl; ○, without hemin Cl; ▲, without hemin Cl but with 0.5 pmol of RF added at 0 min or at 10 min (×, arrow). RF stimulated protein synthesis catalytically; 10–20 pmol of globin was synthesized in 30 min for each pmol of RF added. Specific activity of this preparation was 48,000 units/mg of protein, where 1 unit is the amount of RF required to restore protein synthesis by 50% in heme-deficient lysates. When 1 unit is defined as the amount of RF required to stimulate (eIF-2·GDP) complex formation by 50%, then specific activity was 10,000 units/mg of protein. (*C*) Binary complex formation. Assay mixtures (20 μl) contained 20 mM Tris·HCl (pH 7.6), 100 mM KCl, 250 μM Mg(OAc)$_2$, 0.5 mM dithiothreitol, creatine kinase at 100 μg/ml, 3 pmol of eIF-2 (80% pure), 2 μM [^{3}H]-GDP (6,500 cpm/pmol), and RF as indicated. Incubation was at 30°C for 10 min in Eppendorf tubes (Sarstedt 72-690). The assay was stopped by the addition of 1 ml of cold buffer [20 mM Tris·HCl, pH 7.6/100 mM KCl/1 mM Mg(OAc)$_2$], and (eIF-2·[^{3}H]GDP) was collected by filtration on Millipore membranes (HAWP 02500) at reduced pressure, each tube was rinsed with three 1-ml portions of buffer, and the filter was washed with two 3.5-ml portions. The membranes were heat dried and radioactivity was determined in 5 ml of Econofluor (New England Nuclear). RF acted catalytically; at half-maximal activity, 1 pmol of RF catalyzed the formation of 18 pmol of (eIF-2·[^{3}H]GDP). In the absence of added RF, approximately 0.45 pmol of binary complex was formed.

Assays of RF. RF activity was measured by (*i*) ability to stimulate protein synthesis in heme-deficient reticulocyte lysates and (*ii*) ability to stimulate the formation and dissociation of labeled binary complex (eIF-2·[^{3}H]GDP) as described in Fig. 1. Ternary complex (eIF-2·Met-tRNA$_f$·GTP) formation is described in the legend to Table 1; analysis was the same as for the binary complex (see Fig. 1).

Materials. ATP, GTP, GDP, creatine kinase, and hexokinase were purchased from Sigma. [8-^{3}H]GDP (9 Ci/mmol; 1 Ci = 37 GBq), [γ-^{32}P]ATP (10–40 Ci/mmol), and [^{14}C]leucine (338 mCi/mmol) were purchased from New England Nuclear. [^{3}H]-Met-tRNA$_f$ (13,000 cpm/pmol) and unlabeled Met-tRNA$_f$ were provided by Vivian Ernst (Brandeis University).

RESULTS AND DISCUSSION

Effect of RF and Mg^{2+} on Binary Complex Formation. The kinetics of binary complex (eIF-2·[^{3}H]GDP) formation was examined in the presence and absence of RF and Mg^{2+} (Fig. 2). In the presence of 250 μM Mg^{2+} but no added RF, the binding of [^{3}H]GDP to eIF-2 was rapid but limited (0.55 pmol). Further addition of 0.2 pmol of RF stimulated binary complex formation 3.3-fold (Fig. 2*A*); at half-maximal stimulation, ≈18 pmol of binary complex was formed per pmol of RF. In the absence of both Mg^{2+} and RF, a similar amount of binary complex (1.8 pmol) was formed (Fig. 2*A*). Hence, as noted before (16), physiological concentrations of Mg^{2+} limit binary complex formation. RF overcomes this effect of Mg^{2+} and catalyzes binary complex formation to a level similar to that achieved by the spontaneous association of eIF-2 and GDP in the absence of Mg^{2+} and RF.

Addition of excess unlabeled GDP (°GDP) to the (eIF-2·[^{3}H]-GDP) complex (0.55 pmol) formed in the presence of Mg^{2+} alone produced little or no chase of [^{3}H]GDP from the complex, but further addition of 0.2 pmol of RF catalyzed a rapid exchange (Fig. 2*B*). Similarly, the [^{3}H]GDP moiety in the binary complex (1.8 pmol) formed in the presence of both Mg^{2+} and RF undergoes a rapid exchange with added °GDP (Fig. 2*B*). The [^{3}H]GDP moiety in the binary complex (1.8 pmol) formed spontaneously in the absence of both Mg^{2+} and RF (Fig. 2*C*) is also rapidly chased by excess °GDP, but this chase can be blocked if Mg^{2+} (250 μM) is added after complex formation but prior to °GDP addition (Fig. 2*C*). These data support the con-

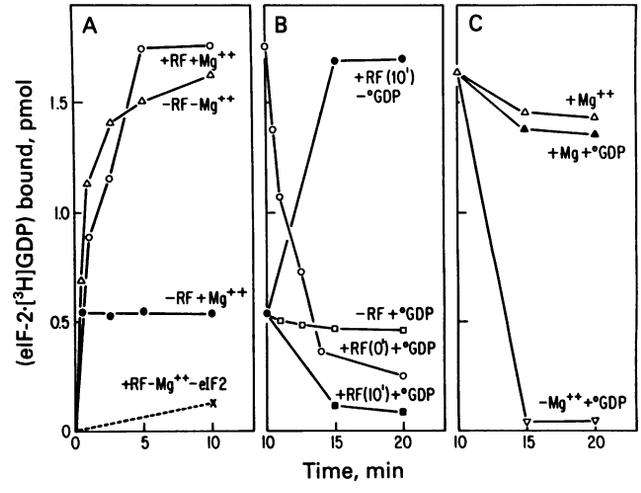

FIG. 2. Kinetics of (eIF-2·[^{3}H]GDP) formation and dissociation: Role of Mg^{2+}. Binary complex-formation assays (20 μl) were carried out over time as described in Fig. 1, except as noted. In all curves, each point represents a separate assay. (*A*) Formation. ●, Without RF; ○, with 0.2 pmol of RF; △, without both RF and Mg(OAc)$_2$; ×, with 0.2 pmol of RF but without both eIF-2 and Mg(OAc)$_2$. (*B*) Dissociation. (eIF-2·[^{3}H]GDP) was allowed to form in a standard 10-min incubation at 30°C in the absence of RF (●) (0.55 pmol of complex) and in the presence of 0.2 pmol of RF (○) (1.8 pmol of complex). At 10 min, additions to incubation mixtures were □, 40 μM unlabeled GDP (°GDP); ■, 0.2 pmol of RF and 40 μM °GDP; ●, 0.2 pmol of RF; ○, 40 μM °GDP. (*C*) Effect of Mg^{2+}. (eIF-2·[^{3}H]GDP) was allowed to form (1.7 pmol) at 30°C for 10 min in the absence of both RF and Mg^{2+}. At 10 min, additions were ▽, 40 μM °GDP; △, 250 μM Mg(OAc)$_2$; ▲, 250 μM Mg(OAc)$_2$/40 μM °GDP.

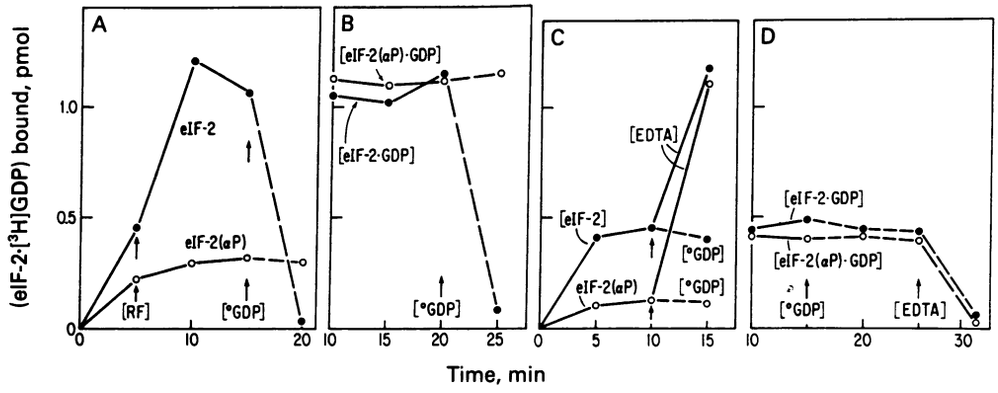

FIG. 3. Effect of HRI on RF-mediated (eIF-2·[³H]GDP) formation and °GDP exchange. Binary complex formation (in 20 μl) was assayed as described in Fig. 1. Where indicated, eIF-2α phosphorylation, °GDP exchange, and EDTA treatment were carried out in a stepwise manner. Standard incubation mixtures (20 μl) contained 20 mM Tris·HCl (pH 7.6), 100 mM KCl, 500 μM $Mg(OAc)_2$, 100 μM creatine phosphate, creatine kinase (100 μg/ml), 0.5 μg of purified HRI (step 7 in ref. 25), and 3 pmol of eIF-2. Other additions and changes were as follows. (*A*) Prephosphorylation of eIF-2 for 10 min in a standard incubation mixture containing 40 μM ATP (○) or H_2O (●). Other additions to both mixtures were 2 μM [³H]GDP (6,500 cpm/pmol) at 0 min, 0.2 pmol of RF at 5 min (arrows), and 40 μM unlabeled GDP (°GDP) at 15 min. (*B*) (eIF-2·[³H]GDP) (1.1–1.2 pmol) was allowed to form for 10 min in the standard incubation mixture by the addition at 0 min of 2 μM [³H]GDP (6,500 cpm/pmol) and 0.2 pmol of RF. Subsequent additions were 40 μM ATP (○) or H_2O (●) at 10 min and 40 μM °GDP at 20 min. The rate and extent of phosphorylation of eIF-2α in the binary complex was the same as for free eIF-2. (*C*) Phosphorylation of eIF-2α for 10 min in a standard incubation mixture containing 40 μM ATP (○) or H_2O (●). Other additions to both mixtures were 2 μM [³H]GDP at 0 min and 40 μM °GDP or 1 mM EDTA at 10 min. (*D*) (eIF-2·[³H]GDP) (0.45 pmol) was allowed to form for 10 min in standard incubation mixtures by the addition at 0 min of 2 μM [³H]GDP (6,500 cpm/pmol) but no RF. Subsequent additions were 40 μM ATP (○) or H_2O (●) at 10 min, 40 μM °GDP to both mixtures at 15 min, and 1 mM EDTA to both at 25 min. Controls containing no HRI and no ATP or containing ATP alone gave results identical to those containing HRI but no ATP and are not included in the figure.

clusion that Mg^{2+} stabilizes the binary complex and that RF is required to catalyze the exchange with added °GDP.

Effect of Phosphorylation of eIF-2α on Binary Complex Formation and Dissociation. The phosphorylation of eIF-2 by HRI inhibits both the formation and the dissociation of the binary complex (Fig. 3). Phosphorylated eIF-2 markedly diminished the rate and extent of RF-mediated (eIF-2·[³H]GDP) formation, as well as the RF-catalyzed chase of complexed [³H]GDP by unlabeled GDP (Fig. 3*A*). Phosphorylation of eIF-2 also inhibited formation of the binary complex formed with Mg^{2+} alone (Fig. 3*C*). In examining this further, we found that the eIF-2α moiety in the (eIF-2·GDP) complex was efficiently phosphorylated by HRI and that this blocked the ability of RF to catalyze an exchange with unlabeled GDP (Fig. 3*B*). Of interest was the finding that phosphorylated eIF-2 could form a complex with GDP when Mg^{2+} was removed by chelation with EDTA (Fig. 3*C*). Similarly, [³H]GDP in the phosphorylated binary complex is rapidly chased only when EDTA is also added (Fig. 3*D*). These data show that, in the presence of Mg^{2+} at physiological concentrations, RF is essential for the dissociation of GDP from the binary complex and that direct phosphorylation of the binary complex inhibits this effect of RF. Under nonphysiological

Table 1. Effect of RF on the conversion of binary complex to ternary complex: Inhibition by HRI

Additions			Binary complex, pmol	Ternary complex, pmol
Step 1	Step 2	Step 3		
eIF-2 and GDP			0.31	
eIF-2 and GDP		Met-$tRNA_f$ and GTP	0.31	0.11
eIF-2, GDP, and RF			1.30	
eIF-2, GDP, and RF		Met-$tRNA_f$ and GTP	0.13	1.07
eIF-2, GDP, and RF	HRI		1.28	
eIF-2, GDP, and RF	HRI	Met-$tRNA_f$ and GTP	0.36	0.97
eIF-2, GDP, and RF	HRI and ATP		1.37	
eIF-2, GDP, and RF	HRI and ATP	Met-$tRNA_f$ and GTP	1.18	0.18
eIF-2		Met-$tRNA_f$ and GTP		0.51
eIF-2 and RF		Met-$tRNA_f$ and GTP		1.20
eIF-2	HRI and ATP	Met-$tRNA_f$ and GTP		0.15
eIF-2	HRI and ATP	Met-$tRNA_f$, GTP, and RF		0.21

Assays were carried out at 30°C in three steps as indicated and complexes were analyzed as described in Fig. 1 and in *Methods and Materials*. Step 1 assay mixtures (15 μl) contained 20 mM Tris·HCl (pH 7.6), 100 mM KCl, 500 μM $Mg(OAc)_2$, 0.5 mM dithiothreitol, creatine phosphokinase at 100 μg/ml, 100 μM creatine phosphate, 3 pmol of eIF-2, and 0.5 μM [³H]GDP (for binary complex assays) or 0.5 μM unlabeled GDP (for ternary complex assays); 0.2 pmol of RF was added where indicated. Incubation was for 10 min. Step 2 phosphorylation assay (17 μl) additions were 40 μM ATP and 0.5 μg HRI where indicated. Incubation was for 10 min. Step 3 ternary complex assay (20 μl) additions were 10 μM GTP and 3 pmol of [³H]Met-$tRNA_f$ (13,000 cpm/pmol) where indicated; when binary complex was measured, unlabeled Met-$tRNA_f$ (3 pmol) was added. In one set of four ternary complex assays, no labeled or unlabeled GDP was added, but all other components were added at the same concentrations and in a stepwise manner as shown.

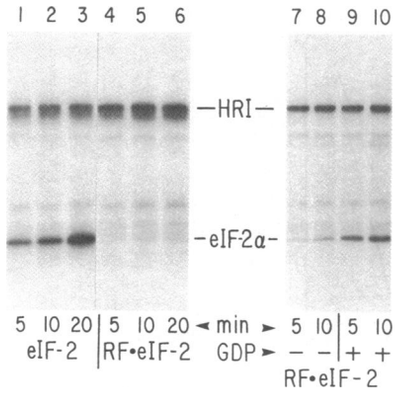

FIG. 4. Effect of RF and GDP on eIF-2α phosphorylation. *In vitro* phosphorylation of eIF-2α was carried out using purified components in reaction mixtures (20 μl) containing 10 mM Hepes (pH 7.4), 1 mM $Mg(OAc)_2$, 40 μM [γ-^{32}P]ATP (10–20 Ci/mmol), 50 mM KCl, and 0.05 μg of HRI. Other additions were 0.5 pmol of eIF-2 (lanes 1–3), 0.5 pmol of RF (lanes 4–10), and 2 μM GDP (lanes 9–10). Incubations were carried out at 30°C for the indicated periods and reactions were terminated by the addition of protein dissociation buffer. Samples were heated for 2.5 min in a boiling water bath, electrophoresed in a 0.1% $NaDodSO_4$/10% acrylamide/0.26% bisacrylamide gel, and then stained and dried as described previously (1). The figure is an autoradiogram.

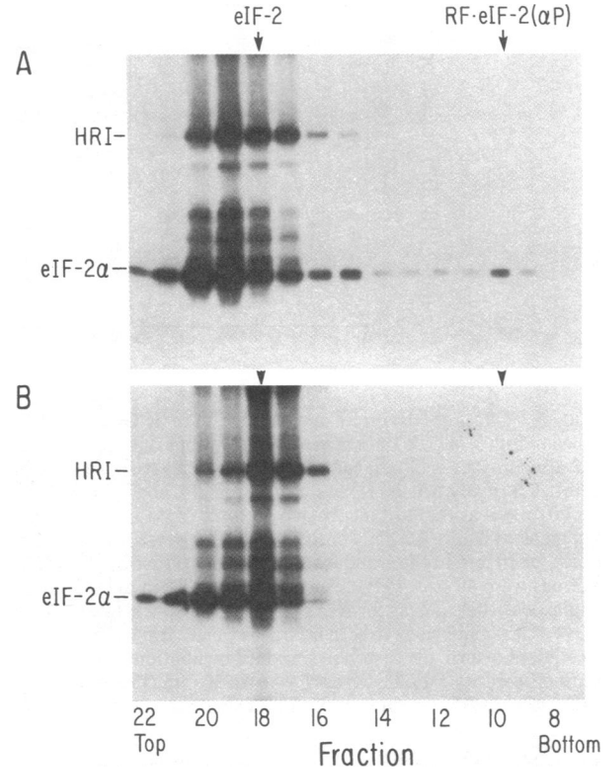

FIG. 5. Formation of a [RF·eIF-2(αP)] complex in the presence of GDP. Reactions were carried out in four steps. Step 1: binary complex incubation mixtures (18 μl) contained 20 mM Tris·HCl (pH 7.6), creatine kinase at 100 μg/ml (as carrier), 500 μM $Mg(OAc)_2$, 2 mM dithiothreitol, 20 μM [^{32}P]ATP (10–20 Ci/mmol), 2.5 pmol of eIF-2, and 0.6 pmol of RF. Incubation was for 10 min at 30°C in the presence (*A*) or absence (*B*) of 40 μM GDP. Step 2: for phosphorylation of eIF-2, all incubation mixtures (19 μl) were supplemented with 0.5 μg of HRI. Complexed eIF-2 (*A*) and free eIF-2 (*B*) were phosphorylated for 10 min at 30°C. Step 3: excess ATP was hydrolyzed by the addition to all assay mixtures (21 μl) of 0.25 unit of hexokinase and 5 mM glucose followed by incubation for 7 min at 30°C. Step 4: all assay mixtures (22 μl) were supplemented with 5 pmol of eIF-2 and incubated for 7 min at 30°C to ensure the presence of excess unphosphorylated (eIF-2·GDP) (*A*) or free eIF-2 (*B*). Reaction mixtures were chilled and layered on a 5-ml 15–50% linear glycerol gradient containing 20 mM Tris·HCl (pH 7.5), 100 mM KCl, 500 μM $Mg(OAc)_2$, and 2 mM dithiothreitol. The tubes were centrifuged at 45,000 rpm for 18 hr at 2°C in a Beckman SW 50.1 rotor, and 0.2-ml fractions were collected and supplemented with carrier creatine kinase (10 μg), ovalbumin (5 μg), and bovine serum albumin (5 μg). Fractions were brought to 0.25 M NaOAc (pH 5.5) and protein was precipitated by the addition of 2 vol of absolute ethanol. After 20 hr at −20°C, precipitates were collected by centrifugation, dissolved in sample buffer, and electrophoresed, and the gels were stained and dried as described (1). [^{32}P]Phosphoprotein profiles were developed by autoradiography.

conditions (absence of Mg^{2+}), eIF-2 and GDP spontaneously form a complex but its formation and dissociation are neither dependent on RF nor affected by the phosphorylation of eIF-2α.

Effect of RF on the Conversion of Binary Complex to Ternary Complex: Inhibition by HRI. The strong affinity between eIF-2 and GDP probably accounts for the potent inhibition of ternary complex formation by GDP (21). Since RF catalyzes the dissociation of GDP from eIF-2, the role of RF in the formation of the ternary complex (eIF-2·Met-tRNA$_f$·GTP) from the binary complex [eIF-2·GDP] was examined. As shown in Table 1, addition of Met-tRNA$_f$ and GTP to the binary complex formed in the presence of Mg^{2+} alone did not change the level of binary complex (0.31 pmol) and produced little ternary complex (0.11 pmol). If RF was also added, >80% of the binary complex formed (1.3 pmol) was converted to ternary complex (1.07 pmol). In accord with other studies (16, 26), this finding indicates that, even in the presence of excess GDP, RF promotes ternary complex formation by catalyzing the exchange of GTP for GDP. When the (eIF-2·GDP) complex was phosphorylated by the addition of HRI and ATP, there was marked inhibition of RF-catalyzed dissociation of binary complex and its conversion to ternary complex (Table 1). There is some indication that RF may promote ternary complex formation directly from its components in the absence of GDP (Table 1). This effect may be a specific property of RF, but it is possible that some GDP is generated from the GTP during the incubation and that RF relieves the resultant inhibition and thus stimulates ternary complex formation.

Kinetics of Phosphorylation of eIF-2α Contained in RF. We next examined the effect of RF on the phosphorylation of eIF-2α by HRI. Free eIF-2 was found to be phosphorylated by HRI at a rate 10 times that of eIF-2 complexed with RF (Fig. 4). However, in the presence of added GDP, the rate of phosphorylation of eIF-2α complexed to RF was stimulated 6- to 10-fold—i.e., to approximately the rate of phosphorylation of free eIF-2. This effect is probably the result of GDP-promoted dissociation of RF·eIF-2 followed by the rapid formation of (eIF-2·GDP), a complex that is phosphorylated as efficiently as free eIF-2. Alternatively, GDP may promote a conformational change in RF in which bound eIF-2α becomes available for phosphorylation. Although our data favor the first model, we cannot at present rule out the alternative.

Interaction of RF and [eIF-2(αP)·GDP]. The observation that catalytic levels of added RF restore protein synthesis in heme-deficient lysates suggests that, in heme deficiency, endogenous RF is nonfunctional. Our *in vitro* studies show that, in the presence of GDP, RF can react with binary complex containing phosphorylated eIF-2 to form a [RF·eIF-2(αP)] complex (Fig. 5). Safer *et al.* (26) recently reported that RF can bind to phosphorylated eIF-2 in the presence of GTP and GDP but not in their absence. The data in Fig. 6 indicate that, in the presence of GDP and RF, the phosphorylation of increasing levels of (eIF-2·GDP) leads to increased formation of [RF·eIF-2(αP)].

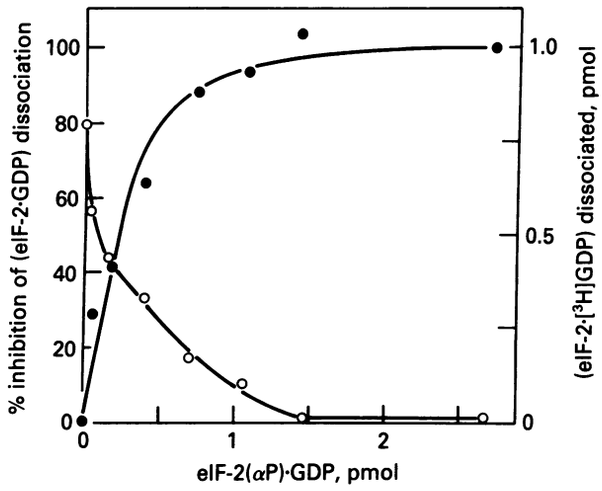

FIG. 6. Inhibition of RF-mediated dissociation of (eIF-2·[^{3}H]GDP) by (eIF-2(αP)·GDP]. Reactions were carried out in 4 steps. Step 1: binary complex assay mixtures (8 μl) contained 20 mM Tris·HCl (pH 7.6), 100 mM KCl, 500 μM $Mg(OAc)_2$, 2 mM dithiothreitol, creatine kinase at 100 μg/ml (as carrier), 40 μM ATP, 40 μM GDP, 0.1 pmol of RF, and 0–5.5 pmol of eIF-2. Incubation was for 10 min at 30°C. Step 2: for phosphorylation of binary complex and free eIF-2, all assay mixtures (9 μl) were supplemented with 0.5 μg of HRI and incubated for 10 min at 30°C; under these conditions, all free and complexed eIF-2 was phosphorylated. Step 3: to remove excess ATP, all assay mixtures (11 μl) were supplemented with 0.25 unit of hexokinase and 5 mM glucose and incubated for 7 min at 30°C. Values for the amount of [eIF-2(αP)·GDP] in each assay tube were determined by duplicate assays in which [^{3}H]-GDP was added in step 1 and carried through step 3. Step 4: dissociation of binary complex (21 μl of assay mixture) was measured by the addition to each tube of a 10-μl incubation mixture containing excess free eIF-2 (9 pmol) and 1 pmol of unphosphorylated (eIF-2·[^{3}H]GDP) prepared with Mg^{2+} but not RF (Figs. 1 and 2). Incubation was 7 min at 30°C. Labeled binary complex was assayed as in Fig. 1. Values are plotted as percent inhibition of dissociation (●) and as pmol of (eIF-2·[^{3}H]-GDP) dissociated (○) in step 4. Control assays in which HRI was omitted in step 2, carried through all four steps, yielded maximal dissociation (0.8 pmol) of (eIF-2·[^{3}H]GDP) (added in step 4). If RF is omitted in step 1, no dissociation occurs in step 4.

This complex is not readily dissociated (Fig. 6) and consequently its RF component is unavailable to catalyze the dissociation of binary complex.

As depicted in the schema of RF function (Fig. 7), we propose the following. (*i*) Binary complex (eIF-2·GDP) is released during the joining of the 48S complex and the 60S ribosomal subunit. (*ii*) The critical role of RF is to catalyze the dissociation of (eIF-2·GDP) to permit formation of the ternary complex (eIF-2·Met-tRNA$_f$·GTP). (*iii*) (eIF-2·GDP) is the primary site of HRI action. (*iv*) RF interacts with phosphorylated binary complex to form a [RF·eIF-2(αP)] complex that effectively sequesters RF so that it cannot function in the recycling of eIF-2. Because RF is present in lysates in limiting concentration relative to eIF-2, this mechanism would explain how the phosphorylation of 30–40% of the eIF-2 in heme-deficient cells and lysates (27) can inhibit protein synthesis.

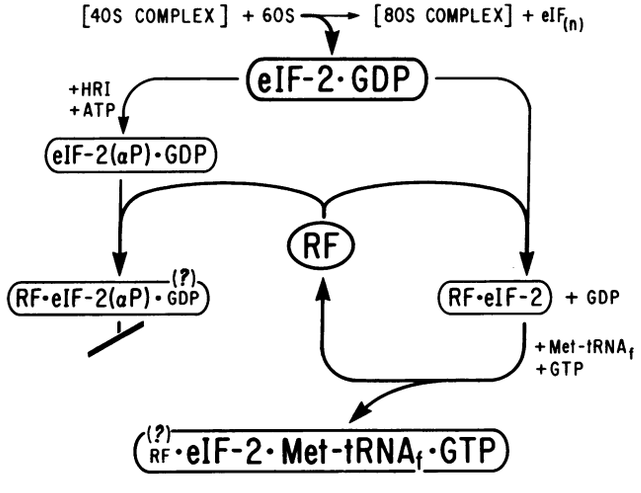

FIG. 7. Proposed schema of RF function.

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