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Binding and release of eukaryotic initiation factor eIF-2 and GTP during protein synthesis initiation*

(phosphorylation of Met-tRNA$_f$ binding factor/GTP hydrolysis/protein synthesis control)

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ABSTRACT **The eukaryotic initiation factor eIF-2 forms a ternary complex with Met-tRNA$_f$ and GTP. This complex binds to the 40S ribosomal subunit in the absence of mRNA and mRNA binding factors. Highly purified eIF-2 from rabbit reticulocytes was labeled with ^{125}I by using the Bolton–Hunter reagent or with [γ-^{32}P]ATP by using the heme-regulated translational inhibitor protein kinase. The labeled eIF-2 was bound, together with equimolar amounts of Met-tRNA$_f$ and GTP, to the 40S subunit. In the presence of mRNA, mRNA binding factors, and 60S ribosomal subunits (complete intiation assay), eIF-2 was released from the 40S initiation complex in the subunit joining reaction. GTP also was released in this step and probably was hydrolyzed in a reaction that is dependent upon eIF-5 and the 60S subunit. The function of phosphorylated eIF-2 in initiation of protein synthesis is discussed.**

The formation of the eukaryotic ternary complex of initiation factor (IF) eIF-2,‡ Met-tRNA$_f$, and GTP is the first step in the *in vitro* sequential assembly of the protein synthesis initiation complex (1, 3, 7). Evidence obtained with crude reticulocyte lysates (8, 9) as well as with fractionated systems (4, 7) suggests that the binding of the ternary complex to the 40S ribosomal subunit precedes the insertion of mRNA. Recently, utilizing a reconstituted system with highly purified components, we have demonstrated that the binding of the ternary complex to the 40S subunit is a prerequisite for globin mRNA binding (10, 11). The insertion of mRNA required eIF-3, eIF-4A, and eIF-4B. The next step in the initiation sequence involved the joining of the 60S subunit to the 40S initiation complex, a step that is mediated by eIF-5 (12). It has been presumed that, during this step, some or perhaps all of the factors are released from the initiation complex, which permits a new round of initiation (recycling). However, the release of IFs has not been demonstrated.

In this report we show that eIF-2 labeled with either ^{32}P or ^{125}I binds to initiation complexes and is released during the joining step. The data also suggest that, in the reconstituted initiation system used in these studies, there is no significant functional difference betwen the phosphorylated and the unphosphorylated eIF-2. In addition, we present further evidence that suggests that the GTP moiety, which enters initiation as a part of the ternary complex, is hydrolyzed and released during the joining of the 40S complex with the 60S subunit (7, 13).

MATERIALS AND METHODS

Purification of eIF-2 and Other Initiation Factors. The eIF-2 was purified >90% from rabbit reticulocyte ribosomes according to the procedure of Schreier *et al.* (14). The concentration of eIF-2 was determined by using the extinction coefficient $E^{1\%}_{1\,cm} = 7.60$ (5); 1 μg of eIF-2 $\simeq$ 7 pmol, assuming a molecular weight of 140,000.

Other IFs were purified as described (14).

Labeling of eIF-2 with ^{125}I. The reaction mixture (150 μl) contained the following components: 270 μg of eIF-2, 30 mM potassium phosphate buffer (pH 7.0), 100 mM KCl, 0.1 mM EDTA, 14 mM 2-mercaptoethanol, 10% glycerol, and 0.1 mCi of ^{125}I-labeled *N*-succimidyl-3-(4-hydroxyphenyl) propionate (Bolton–Hunter reagent) (15); incubation was for 40 min at 0°. The reaction mixture was then passed through a Sephadex G-75 column (0.5 × 6 cm) in 20 mM Tris·HCl, pH 7.6/100 mM KCl/0.1 mM EDTA/14 mM 2-mercaptoethanol/10% glycerol; 50-μl fractions were collected. The ^{125}I-labeled factor eluted with the void volume and was free of unreacted reagent.

Labeling of eIF-2 with [γ-^{32}P]ATP. The reaction mixture contained 125 μg of eIF-2 and 30 μl of the heme-regulated inhibitor§ (a cyclic AMP-independent protein kinase preparation from rabbit reticulocyte lysate, kindly provided by Tim Hunt, Cambridge University, England) in 20 mM Tris·HCl, pH 7.6/100 mM KCl/1.5 mM Mg(OAc)$_2$/0.28 mM [γ-^{32}P]ATP (3.18 Ci/mmol). Incubation was for 5 min at 37°. The mixture was then passed through Sephadex G-75 and ^{32}P-labeled eIF-2 was collected as described above. HRI remained in the eIF-2 preparation.

Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed in 10% slab gels according to the procedure of Laemmli (18). When radioactivity was to be determined in specific bands of the stained gels, the gel was frozen and the bands were then cut out. The gel slices were digested with 1 ml of 30% H_2O_2 at 37° overnight. After addition of 10 ml of toluene-based scintillation fluid (14), the samples were assayed for radioactivity in a Packard liquid scintillation counter (for ^{32}P) or in a Packard gamma scintillation counter (for ^{125}I). For double label counting (^{3}H + ^{125}I), the ^{3}H radioactivity was corrected for the β radiation of ^{125}I.

Binding of eIF-2 to the 40S Ribosomal Subunit. The reac-

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Abbreviations: IF, initiation factor; HRI, heme-regulated translational inhibitor.

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‡ eIF-2 is the designation for the Met-tRNA$_f$ binding factor adopted at the Fogarty International Conference on the Regulation of Protein Synthesis, October 1976 at the National Institutes of Health (Bethesda, MD). Previous designations are as follows: IF-1 (1, 2), IF-L$_3$ (3), IF-E$_2$ (4), IF-MP (5), and EIF-3 (6).

§ This inhibitor, first described by Rabinovitz *et al.* (16) and called "hemin-controlled repressor (HCR)" has been extensively purified by Ranu and London (17) who prefer the term "heme-regulated inhibitor (HRI)."

Table 1. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis

Subunit, $M_r \times 10^{-3}$	^{125}I-Labeled eIF-2		^{32}P-Labeled eIF-2	
	cpm	%	cpm	%
35	82,224	14	39,485	88
50	255,935	43.3	1,013	
55	216,970	36.7		2.2
Rest of gel	35,433	6	4,341	9.8

Purified eIF-2 was labeled with ^{125}I or [γ-^{32}P]ATP. Radioactivity in polypeptides belonging to eIF-2 and in contaminants was determined.

tion mixtures contained the following components in a final volume of 100 μl: 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.3), 100 mM KCl, 0.08 mM GTP, 30 pmol of [^{3}H]Met-tRNA$_f$ (4500 cpm/pmol), 7 pmol of 40S subunits (0.11 A_{260} unit), Mg(OAc)$_2$, and eIF-2 as indicated in the figure legends. Incubation was for 10 min at 37°. The reaction mixtures were then analyzed on 5–29% convex exponential sucrose gradients (3.8 ml) in 20 mM Tris·HCl, pH 7.6/100 mM KCl/8 mM Mg(OAc)$_2$. Centrifugation was at 55,000 rpm in a Spinco SW 56 Ti rotor for 2 hr at 4°. The gradients were collected through a UV-monitoring flow cell system directly into counting vials containing scintillation fluid (11). Radioactivity was determined as described above.

***In Vitro* Formation of 40S and 80S Initiation Complexes.** The preparation of ribosomal subunits, 9S globin mRNA, IFs, and [^{3}H]Met-tRNA$_f$ has been described (14). 9S globin mRNA was labeled with ^{125}I as described (11). The reaction mixture (150 μl) contained the following components: 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.3), 110 mM KCl, 0.5 mM ATP (ATP and GTP solutions contained equimolar amounts of Mg(OAc)$_2$ to compensate for the chelating effect of ATP and GTP), 0.3 μg of eIF-1, 9 μg of eIF-3, 6 μg of eIF-4A, 4.5 μg of eIF-4B, 0.3 μg of eIF-4C, 0.3 μg of eIF-5, and Mg(OAc)$_2$, GTP, Met-tRNA$_f$, 9S globin mRNA, ribosomal subunits, and eIF-2 as indicated in the figure legends. Incubation was for 10 min at 37°. The reaction mixtures were analyzed on 5–35% sucrose gradients (12 ml) as described above. Centrifugation was at 40,000 rpm in the Spinco SW 41 Ti rotor for 3.25 hr at 4°.

RESULTS

Labeling of eIF-2. eIF-2 is composed of three polypeptide chains of approximate molecular weights 55,000, 50,000, and 35,000 (10, 11). The eIF-2 preparation used in these experiments was >90% pure based on its banding profile in sodium dodecyl sulfate/polyacrylamide gels as well as the proportion of radioactivity contained in eIF-2 in preparations labeled with ^{32}P or ^{125}I (Table 1).

Iodination with the Bolton–Hunter reagent results in the modification of -NH$_2$ groups (15). The iodinated eIF-2 was labeled in all three subunits (Table 1). The factor was labeled with ^{32}P by incubation with HRI, the protein kinase isolated from heme-deficient rabbit reticulocyte lysates (19). Incubation with HRI in the presence of [^{32}P]ATP leads to the phosphorylation of a hydroxyl residue of serine in the smallest subunit of eIF-2. This enzymatic modification is specific for eIF-2 and is thought to be involved in the regulation of polypeptide chain initiation (19). The phosphorylated factor was significantly labeled only in the smallest subunit; about 97% of the total ^{32}P incorporated in eIF-2 was in the 35,000-dalton subunit.

The phosphorylated eIF-2 preparation contained slightly more labeled contaminants than the iodinated preparation (Table 1). This is probably due to autophosphorylation of the HRI components utilized in the phosphorylation reaction (19).

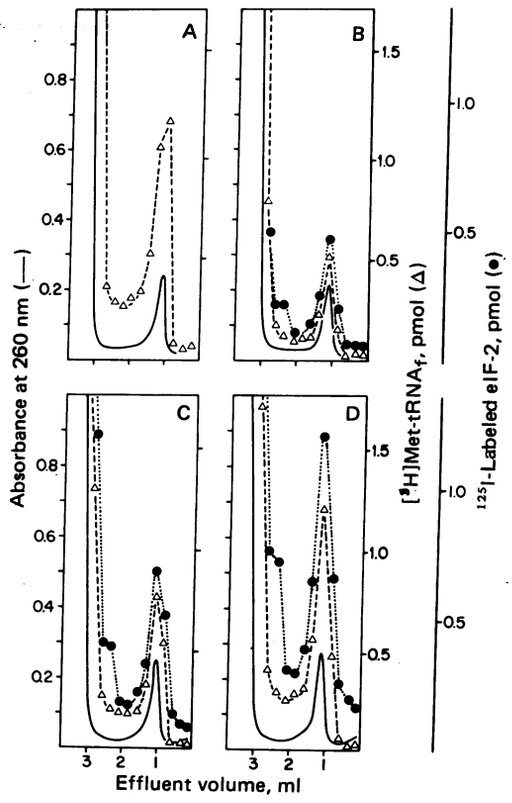

FIG. 1. Binding of the ternary complex (^{125}I-labeled eIF-2, GTP, Met-tRNA$_f$) to the 40S subunit. Assays contained 7 pmol of 40S ribosomal subunits and 3.7 μg of unlabeled eIF-2 (*A*), 2 μg of ^{125}I-labeled eIF-2 (*B*), 3 μg of ^{125}I-labeled eIF-2 (*C*), or 5 μg of ^{125}I-labeled eIF-2 (*D*). The magnesium concentration was 2.9 mM. [^{3}H]Met-tRNA$_f$, 4500 cpm/pmol; ^{125}I-labeled eIF-2, 5900 cpm/pmol.

In calculating the specific activities of the two labeled eIF-2 preparations, the following assumptions were made: (*i*) the eIF-2 preparations are 90% pure; (*ii*) the native molecular weight of eIF-2 is 140,000 (55,000 + 50,000 + 35,000).

Binding of eIF-2 to the 40S Subunit. In the presence of GTP and Met-tRNA$_f$, eIF-2 forms the ternary complex [eIF-2, GTP, Met-tRNA$_f$] which then binds to the 40S ribosomal subunit. This reaction is mRNA-independent (4, 11). Fig. 1 shows the extent of binding of the ternary complex containing ^{125}I-labeled eIF-2 to the 40S subunit. In this experiment the ternary complex was bound to the 40S subunit in the absence of mRNA and mRNA binding factors at 2.9 mM Mg(OAc)$_2$ [high magnesium concentrations stabilize the (40S, eIF-2, GTP, Met-tRNA$_f$) complex (unpublished data)]. The input ^{125}I-labeled eIF-2 was increased from 12.5 pmol (Fig. 1*B*) to 19 pmol (Fig. 1*C*) to 31.5 pmol (Fig. 1*D*). The amount of ternary complex bound to the 40S subunit increased from 1.1 pmol (Fig. 1*B*) to 3.2 pmol (Fig 1*D*). About 5 pmol of 40S subunits was recovered from each gradient as calculated from the absorbance profile. Approximately equimolar amounts of [^{3}H]Met-tRNA$_f$ and ^{125}I-labeled eIF-2 (eIF-2/Met-tRNA$_f$ = 0.96, Fig. 1*D*) were bound to the 40S subunit and up to 60% of the 40S subunits bound the ternary complex (Fig. 1 *A* and *D*). In the experiment shown in Fig. 2, ^{32}P-labeled eIF-2 was used in place of ^{125}I-labeled eIF-2 and similar results were obtained. The GTP analogue β,γ-methyleneguanosine 5'-triphosphate and high magnesium concentrations were used to obtain maximal binding of ternary com-

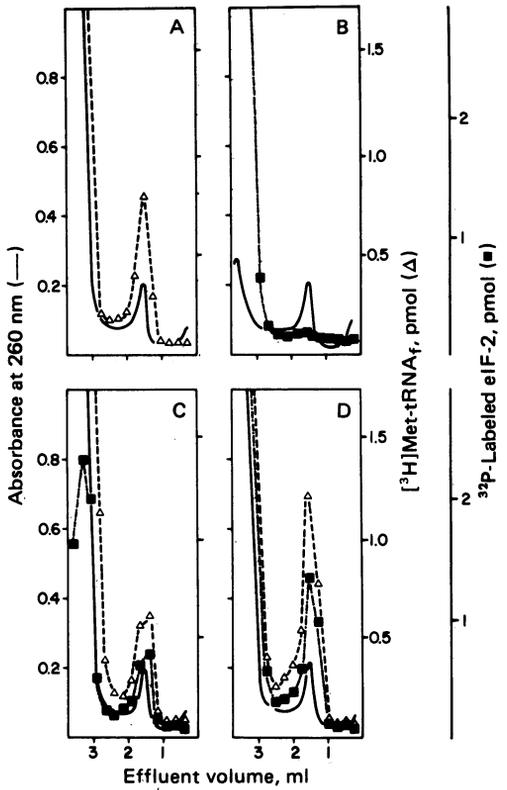

FIG. 2. Binding of the ternary complex (^{32}P-labeled eIF-2, GTP, Met-tRNA$_f$) to the 40S subunit. Assays contained 7 pmol of 40S ribosomal subunits and 1.8 μg of unlabeled eIF-2 (*A*), 5.5 μg of ^{32}P-labeled eIF-2 and no Met-tRNA$_f$ (*B*), 2.75 μg of ^{32}P-labeled eIF-2 (*C*), or 5.5 μg of ^{32}P-labeled eIF-2 (*D*). The magnesium concentration was 7 mM. Instead of GTP, the analogue β,γ-methyleneguanosine 5′-triphosphate was used. [^{3}H]Met-tRNA$_f$, 4500 cpm/pmol; ^{32}P-labeled eIF-2, 2750 cpm/pmol.

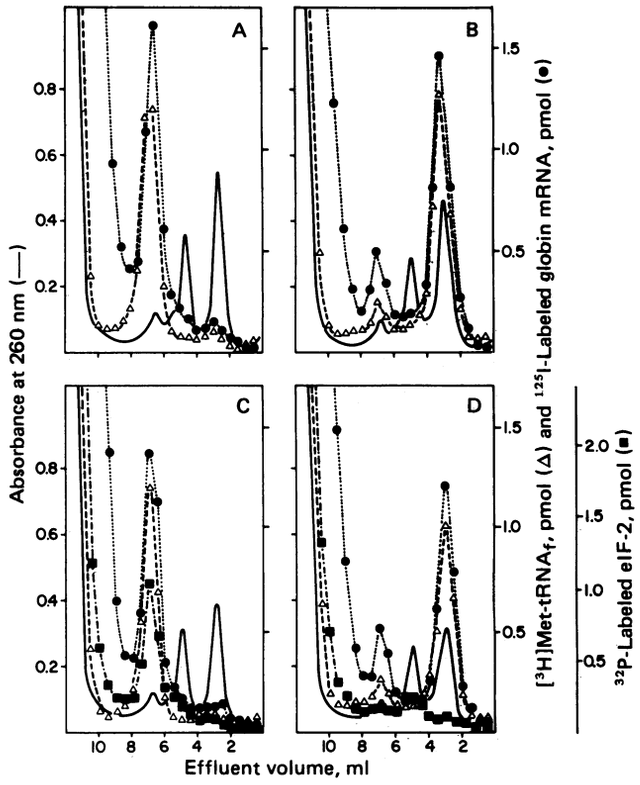

FIG. 3. Release of ^{32}P-labeled eIF-2 during the joining reaction. The reaction mixture (150 μl) contained 16 pmol of 40S and 16 pmol of 60S ribosomal subunits, 1.7 mM $Mg(OAc)_2$, 29 pmol of [^{3}H]Met-tRNA$_f$, and 13 pmol of ^{125}I-labeled 9S globin mRNA. In addition, the reaction mixtures contained other components as follows: (*A*) and (*C*), 0.08 mM β,γ-methyleneguanosine 5′-triphosphate; (*B*) and (*D*), 0.08 mM GTP; (*A*) and (*B*), 5 μg of eIF-2; (*C*) and (*D*), 5 μg of ^{32}P-labeled eIF-2. [^{3}H]Met-tRNA$_f$, 4500 cpm/pmol; ^{125}I-labeled 9S globin mRNA, 4500 cpm/pmol; ^{32}P-labeled eIF-2, 2750 cpm/pmol.

plex to the 40S subunit (unpublished data). As shown in Fig. 2D, 80% of the 40S subunits carried an eIF-2 and a Met-tRNA$_f$ moiety. The eIF-2/Met-tRNA$_f$ ratio on the 40S subunit was 0.90 (Fig. 2*C*) and 0.99 (Fig. 2*D*). In the absence of Met-tRNA$_f$ (Fig. 2*B*), no stable binding of ^{32}P-labeled eIF-2 to the 40S subunit could be detected under the conditions utilized for sucrose gradient analysis.

Release of eIF-2 from Initiation Complexes. Binding of the ternary complex (eIF-2, GTP, Met-tRNA$_f$) to the 40S subunit is a prerequisite for mRNA binding. The insertion of mRNA into the 40S initiation complex is completely dependent on eIF-3, eIF-4A, and eIF-4B and partially dependent on eIF-1 and eIF-4C (11). In addition, ATP is required for the binding of mRNA (10–12). The last step in the formation of the 80S initiation complex is the eIF-5-mediated joining of the 40S initiation complex with the 60S ribosomal subunit (10–12). This step is inhibited by the nonhydrolyzable GTP analogue, β,γ-methyleneguanosine 5′-triphosphate, Fig. 3 *A* and *B*. When ^{32}P-labeled eIF-2 was used in place of unphosphorylated factor, similar results were obtained. The phosphorylated eIF-2 remained bound to the (40S, Met-tRNA$_f$, mRNA) complex (Fig. 3*C*) but was released in the subsequent joining step (Fig. 3*D*). In the 40S initiation complexes (Fig. 3*C*) the mRNA/Met-tRNA$_f$ ratio was 0.88 with the ^{125}I-labeled 9S globin mRNA background subtracted (11), and the eIF-2/Met-tRNA$_f$ ratio was 1. No eIF-2 was bound to the 80S initiation complex.

The same result was obtained when ^{125}I-labeled instead of ^{32}P-labeled eIF-2 was used (Fig. 4). In the experiment shown in Fig. 4*A*, the (40S, eIF-2, GTP, Met-tRNA$_f$) complexes were formed in the absence of mRNA as well as of eIF-4A, eIF-4B, eIF-4C, and eIF-5 (there is no requirement for mRNA and mRNA binding factors for the binding of the ternary complex to the 40S subunit). At this stage, eIF-2 was bound to the 40S subunit. After the addition of mRNA, eIF-4A, eIF-4B, eIF-4C, and eIF-5, the 40S complexes bound mRNA and joined a 60S ribosomal subunit to form the 80S initiation complex; eIF-2 was released in this reaction (Fig. 4*B*). About 50% of the 80S ribosomes carried Met-tRNA$_f$ and 9S globin mRNA in about equimolar amounts (Fig. 3*B*).

Release of GTP during the Joining Reaction. Fig. 5 demonstrates the binding and release of ring-labeled [^{3}H]GTP (Fig. 5 *A–C*) or γ-^{32}P-labeled GTP (Fig. 5 *D–F*) during 40S and 80S initiation complex formation. Both ring- and γ-labeled GTP give similar results: GTP was found in about equimolar amounts with [^{3}H]Met-tRNA$_f$ (Fig. 5 *D* and *E*) or ^{125}I-labeled globin 9S mRNA (Fig. 5*B*) in the 40S initiation complex but not in the 80S initiation complex (Fig. 5 *C* and *F*). The GTP/Met-tRNA$_f$ ratio was 0.9 (Fig. 5*D*) and 1.1 (Fig. 5*E*). In Fig. 5*B*, the GTP/mRNA ratio was close to 1 when the ^{125}I-labeled globin mRNA background was subtracted (11). In Fig. 5*F* the GTP/Met-tRNA$_f$ ratio present on the 40S initiation complexes which remain after the joining step was well below unity. About 35% of the 40S ribosomes (Fig. 5 *D* and *E*) and 40% of the 80S ribosomes (Fig. 5*F*) bound Met-tRNA$_f$. The experiment clearly demonstrates that GTP or its hydrolysis products are released from the ribosome in the same step as eIF-2—namely, during the subunit joining reaction.

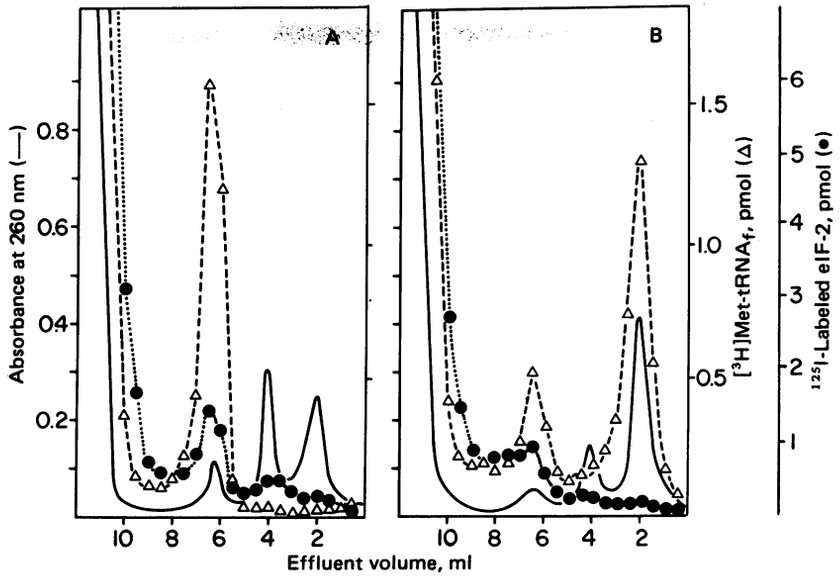

FIG. 4. Release of ^{125}I-labeled eIF-2 during the joining reaction. The reaction mixture contained 11 pmol of 80S ribosomes, 2.7 mM $Mg(OAc)_2$, 0.08 mM GTP, 35 pmol of [^{3}H]Met-tRNA$_f$, 0.05 A_{260} unit of 9S globin mRNA, and 6 μg of ^{125}I-labeled eIF-2. (*A*) Without eIF-4A, eIF-4B, eIF-4C, and eIF-5. (*B*) Complete. [^{3}H]Met-tRNA$_f$, 4500 cpm/pmol; ^{125}I-labeled eIF-2, 1250 cpm/pmol.

DISCUSSION

eIF-2 forms a ternary complex with Met-tRNA$_f$ and GTP in a 1:1 molar ratio (1, 20). Using highly purified eIF-2 labeled with ^{125}I or ^{32}P, together with labeled Met-tRNA$_f$ and labeled GTP, we show directly that eIF-2 is bound in stoichiometric amounts with Met-tRNA$_f$ (Figs. 1 and 2) and GTP (Fig. 5) to the 40S ribosomal subunit. The binding of eIF-2 to the 40S ribosomal subunit is absolutely dependent on the presence of Met-tRNA$_f$ under the conditions of incubation and sucrose gradient centrifugation (Fig. 2*B*), indicating that eIF-2 binds only in the form of the ternary complex (eIF-2, GTP, Met-tRNA$_f$) to the 40S subunit. Both iodinated and phosphorylated eIF-2 are as

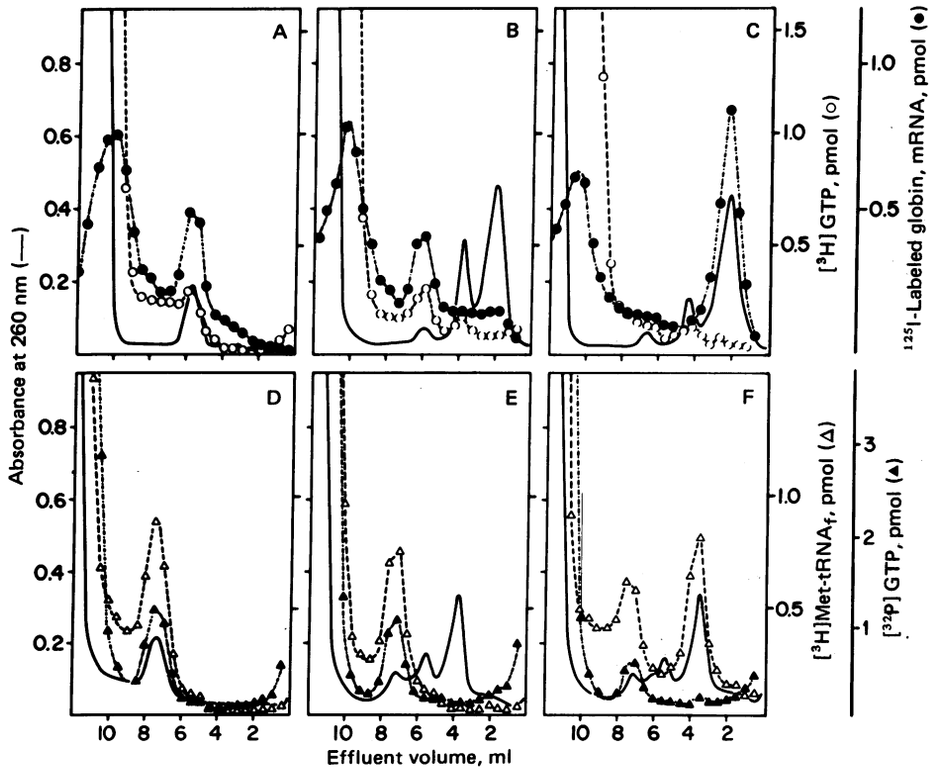

FIG. 5. Binding and release of [^{3}H]GTP and [γ-^{32}P]GTP from initiation complexes. The reaction mixture contained 14 pmol of 40S ribosomal subunits, 1.4 mM $Mg(OAc)_2$, and 5.5 μg of eIF-2. (*Upper*) With 0.01 mM [^{3}H]GTP, 10 pmol of ^{125}I-labeled 9S mRNA, and 30 pmol of unlabeled Met-tRNA$_f$. (*A*) Without 60S subunits and eIF-5; (*B*) without eIF-5 but with 14 pmol of 60S subunits; (*C*) complete system. [^{3}H]GTP 4700 cpm/pmol; ^{125}I-labeled 9S globin mRNA, 5600 cpm/pmol. (*Lower*) With 0.017 mM [γ-^{32}P]GTP, 0.05 A_{260} unit of unlabeled 9S globin mRNA, and 30 pmol of [^{3}H]Met-tRNA$_f$. (*D*) Without 60S subunits and eIF-5; (*E*) without eIF-5 but with 14 pmol of 60S subunits; (*F*) complete system. [γ-^{32}P]GTP, 2000 cpm/pmol; [^{3}H]Met-tRNA$_f$, 4800 cpm/pmol.

active in binding to the 40S subunit as the unlabeled factor (Figs. 1 and 2). The ternary complex binds to the 40S subunit in the absence of mRNA and mRNA binding factors. Both ring-labeled [^{3}H]GTP and γ-^{32}P-labeled GTP bind together with eIF-2 to the 40S subunit but are not found in the 80S initiation complex. The release of GTP requires both the initiation factor eIF-5 and the 60S ribosomal subunit (Fig. 5). This indicates that both eIF-2 and GTP introduced as part of the ternary complex are released in the eIF-5-catalyzed joining reaction. The fact that the nonhydrolyzable GTP analogue methyleneguanosine triphosphate allows 40S initiation complex formation but inhibits completely the formation of 80S initiation complexes (7, 10, 11) (Fig. 3) suggests that GTP is hydrolyzed during the subunit joining reaction.

These and other studies (21) in highly purified systems demonstrate that, under the conditions of reconstitution, the phosphorylated eIF-2 by itself is as efficient as unphosphorylated eIF-2 in promoting partial reactions and in the formation of 40S and 80S initiation complexes. However, it should be emphasized that, in the reconstituted system described here, unphosphorylated or phosphorylated eIF-2 is present in excess relative to the amount of ribosomes. In addition, under the conditions of 40S initiation complex formation (and most likely also of 80S complex formation), there is no reinitiation.

On the other hand, there is convincing evidence that, in lysates under conditions that closely resemble *in vivo* conditions, the phosphorylation of the smallest subunit of eIF-2 plays a significant role in the regulation of protein synthesis (19) (for a review, see ref. 22). One of the characteristics of this inhibition is a depletion of the (40S, Met-tRNA$_f$) complex (23). This phenomenon is also observed in gel-filtered lysates or with crude reticulocyte ribosomes and depends on the presence of both HRI and ATP (19). Moreover, in reconstituted crude systems, no inhibition, by HRI plus ATP, of ternary complex (eIF-2, GTP, Met-tRNA$_f$) formation has been observed but, under the same conditions, the *in vitro* binding of the ternary complex to the 40S subunit has been found to be inhibited (24–26).

One possible explanation for these findings is that there may be additional components required for the efficient binding of the ternary complex to the 40S subunit and these components do not function properly with phosphorylated eIF-2. Alternatively, the phosphorylated eIF-2 may function only stoichiometrically but not catalytically (27) and is essentially inactivated after one round of initiation. This would explain why phosphorylated eIF-2 can function in the *in vitro* assembly of initiation complexes in the purified system but not in the synthesis of globin chains in the reticulocyte lysate system, which depends on the catalytic function of eIF-2.

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