Protein Synthesis in Brine Shrimp Embryos and Rabbit Reticulocytes

THE EFFECT OF Mg²⁺ ON BINARY (EUKARYOTIC INITIATION FACTOR 2-GTP) AND TERNARY (EUKARYOTIC INITIATION FACTOR 2-GTP-MET-tRNAᵢ) COMPLEX FORMATION*

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We have prepared eukaryotic initiation factor 2 (eIF-2) from rabbit reticulocytes and Artemia embryos and studied the effect of Mg²⁺ on binary (eIF-2-GTP) and ternary (eIF-2-GTP-Met-tRNAᵢ) complex formation. Under conditions where Mg²⁺ inhibits Met-tRNAᵢ binding to reticulocyte eIF-2, ternary complex formation with Artemia eIF-2 is not inhibited. Similarly, the formation of eIF-2-GDP with Artemia eIF-2 is stimulated by Mg²⁺, whereas the corresponding reticulocyte binary complex is strongly inhibited. In the presence of 1 mM Mg²⁺, the isolated Artemia eIF-2-GDP complex is stable in the absence of any added nucleotide, but readily exchanges bound GDP for free GTP. However, the reticulocyte eIF-2-GDP complex is significantly more stable in the presence of GTP, and nucleotide exchange is dependent upon the addition of a factor isolated from either the postribosomal supernatant or the high salt wash of rabbit reticulocyte ribosomes. This factor also stimulates Met-tRNAᵢ binding to both Artemia and reticulocyte eIF-2.

The formation of an eIF-2-GTP-Met-tRNAᵢ ternary complex is the first step in the initiation of protein synthesis (1-4). After the transfer of Met-tRNAᵢ into an 80 S initiation complex, GTP is hydrolyzed (5) and presumably a binary complex (eIF-2-GDP) is released into the cytosol (6-7). Recently, a factor was isolated from the postribosomal supernatant of rabbit reticulocytes which stimulates the exchange of GTP for GDP in the isolated eIF-2-GDP complex (7-10). This factor was postulated to permit recycling of eIF-2 in chain initiation. Previous reports from this laboratory (11) have shown that Artemia eIF-2 binds [³H]GDP, and readily exchanges this GTP for added GDP in a two stage assay without isolation of a binary complex.

In this communication, we examine the effect of Mg²⁺ on eIF-2-GDP formation and on Met-tRNAᵢ binding to Artemia and reticulocyte eIF-2. Mg²⁺ stimulates binary and ternary complex formation with Artemia eIF-2 and inhibits these reactions with reticulocyte eIF-2. The binary complex formed

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‡ The abbreviation used is: eIF, eukaryotic initiation factor.

with either initiation factor is stable after isolation by chromatography on phosphocellulose in the presence of 1 mM Mg²⁺. Upon the addition of GTP, nucleotide exchange readily occurs with the Artemia preparation, but not with the reticulocyte binary complex. Nucleotide exchange with the reticulocyte eIF-2-GDP is dependent upon the presence of a factor isolated from the postribosomal supernatant or the high salt wash of rabbit reticulocyte ribosomes. This factor, which has little effect on nucleotide exchange with the Artemia binary complex, stimulates Met-tRNAᵢ binding to both eIF-2 preparations.

EXPERIMENTAL PROCEDURES

Materials—[³H]Methionine (1027 Ci/mmol) and [³H]GDP (10.2 Ci/mmol) were purchased from New England Nuclear. GTP and GDP were obtained from Boehringer Mannheim GmbH. The glycine and bovine serum albumin were from Sigma, and the other specified concentrations (Table I) were prepared as described (11-12).

Preparations—The polypeptide chain initiation factor eIF-2 from Artemia embryos was isolated as described earlier (12). Further purification was achieved by applying 1.4 A₂₆₀ units of this preparation on a DEAE-Tris acryl column (0.8 x 2.0 cm) equilibrated with 50 mM Tris-HCl (pH 7.8), 100 mM KCl, 0.2 mM magnesium acetate, 10 mM β-mercaptoethanol, and 10% glycerol (buffer A). The unadsorbed material (0.9 A₂₆₀ units) was applied to a poly(C)-cellulose column (0.8 x 1.5 cm) previously equilibrated with buffer A. The column was first washed with buffer A and then with buffer A containing 0.3 M KCl. The chain initiation factor was then eluted with buffer containing 0.5 M KCl. The purity of eIF-2 so obtained is approximately 96% as determined by a densitometry scan of the Coomassie blue staining of material subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (12).

Reticulocyte eIF-2 and nucleotide exchange factor were purified by applying ribosomal high salt wash (700 A₂₆₀ units) on a phosphocellulose column (2.5 x 20 cm) previously equilibrated with 20 mM Tris-HCl (pH 7.5), 150 mM KCl, 1 mM dithiothreitol, 0.06 mM EDTA, and 10% glycerol (buffer B). The column was first washed with buffer B and then with buffer B containing 0.45 M KCl and 1.0 M KCl (13). Proteins eluting with 1.0 M KCl (4.2 A₂₆₀ units) were dialyzed against buffer B containing 0.2 M KCl and applied to a CM-Sephadex C-50 column (0.8 x 3.5 cm) equilibrated with buffer B containing 0.2 M KCl. The unadsorbed fraction contained nucleotide exchange activity. Reticulocyte eIF-2 was eluted by washing the column with buffer B containing 0.4 M KCl. The nucleotide exchange factor can also be purified from the postribosomal supernatant by chromatography of this fraction on phosphocellulose and CM-Sephadex C-50.

For the formation of the eIF-2-[³H]GDP binary complex, the 0.75-mL reaction mixture contained 20 mM Tris-HCl (pH 7.8), 100 mM KCl, 750 µg of bovine serum albumin, 1 mM dithiothreitol, 4 µM [³H]GDP (specific activity 5,000-6,000 cpn/µmol), and 80 µg of Artemia eIF-2 or 85 µg of reticulocyte eIF-2. After incubation for 10 min at 30 °C, the reaction mixtures were chilled in ice and magnesium acetate was added to the assay mixture at 1 mM final concentration. Unreacted [³H]GDP was removed by applying the incubation mixtures to a phosphocellulose column equilibrated with buffer B containing 1 mM Mg²⁺. The absorbed binary complex was subsequently eluted with buffer B containing 0.65 M KCl and 1 mM Mg²⁺.

Assays—For measurement of the exchange of free GTP with eIF-2 bound [³H]GDP, the 0.075-mL reaction mixture contained 20 mM Tris-HCl (pH 7.8), 100 mM KCl, 1 mM magnesium acetate, 75 µg of bovine serum albumin, and 1 mM dithiothreitol 0.4-0.7 pmol of eIF-2-[³H]GDP, 200 µM GTP, or other specified concentrations (Table I), 10 µg of nucleotide exchange factor, and 17,000 cpm/µmol of [³H]Met-tRNAᵢ3 or 4 pmol were also present in the assay mixture where indicated. After incubation at 30 or 15 °C, samples were chilled in ice and immediately diluted with 1 ml of cold wash buffer (buffer B).
containing 0.1 M KCl and 1 mM magnesium acetate. The mixture was then filtered through nitrocellulose (S & S, BA-85, 0.45 μm) which was washed, dried, and counted in 8.0 ml of toluene containing 4 g/liter of Omnifluor (New England Nuclear). Other procedures, which include ternary complex formation, polyacrylamide gel electrophoresis in sodium dodecyl sulfate, and preparation of \[^{35}S\]Met-tRNA\(_f\) were as described previously (11–12). Poly(C) was coupled by ultraviolet irradiation to Whatman CF11 cellulose as described elsewhere (14–15).

RESULTS

The formation of a ternary complex ([eIF-2·GTP·Met-tRNA\(_f\)]) by Artemia eIF-2 is stimulated approximately 50% by the addition of 3–4 mM Mg\(^{2+}\) (Fig. 1). In contrast, Mg\(^{2+}\) concentrations as low as 1 mM inhibit ternary complex formation with reticulocyte eIF-2 by about 75% (Figs. 1 and 2). The result is in agreement with reports from other laboratories (16–18).

We noted a similar difference between eIF-2 from Artemia and reticulocytes in the formation of an eIF-2·GDP binary complex. It may be seen in Fig. 1 that the binding of GDP to Artemia eIF-2 is stimulated 2-fold in the presence of 3 mM Mg\(^{2+}\), whereas, under similar conditions, the binary complex formation with reticulocyte eIF-2 is inhibited about 55%.

Earlier studies from our laboratory have indicated that Artemia binds GDP which readily exchanges at 15 and 30°C with exogenously added GTP (11). These experiments were not performed with the isolated binary complex (eIF-2·GDP). Recently, a protein from Ehrlich ascites tumor cells (19) and rabbit reticulocytes (7, 10) was implicated in catalyzing nucleotide exchange from binary complexes containing Ehrlich ascites or reticulocyte eIF-2. This prompted us to examine properties of the isolated binary complex formed with eIF-2 from Artemia and reticulocytes.

We observed that eIF-2·GDP complex from both sources is stable at 30°C in the absence of added nucleotide (Fig. 3). However, the Artemia binary complex readily exchanges bound GDP for free GTP (Fig. 3B). This is in contrast to the reticulocyte eIF-2·[\(^{3}H\)]GDP which is stable in the presence of added GTP, and as seen in Fig. 3B, the nucleotide exchange with reticulocyte eIF-2·GDP is found to be dependent on the presence of a protein factor isolated from the postribosomal supernatant or the high salt wash of rabbit reticulocyte ribosomes. This preparation also relieves Mg\(^{2+}\) inhibition of ternary complex formation by reticulocyte eIF-2 (Fig. 2). This factor has little effect on nucleotide exchange with the Artemia eIF-2·GDP complex. A similar pattern with respect to stability and nucleotide exchange is observed at 15°C with Artemia eIF-2·GDP, except that the exchange occurs at a slower rate (data not shown).

The effect of \[^{35}S\]Met-tRNA\(_f\) on nucleotide exchange and the subsequent formation of an eIF-2·GTP·Met-tRNA\(_f\) ternary complex was examined. As shown in Fig. 4, the addition of Met-tRNA\(_f\) does not influence the rate of nucleotide exchange with eIF-2 from either Artemia or reticulocytes (compare with Fig. 3). Met-tRNA\(_f\) binding to both eIF-2 preparations is increased 2–4-fold by the reticulocyte protein factor.

![Fig. 1. Effect of Mg\(^{2+}\) on ternary and binary complex formation. Assay conditions were as described under "Experimental Procedures." For ternary complex formation, a 75-μl reaction mixture contained 0.8 μg of Artemia eIF-2 or 0.54 μg of reticulocyte eIF-2, 0.2 mM GTP, 3 pmol of \[^{35}S\]Met-tRNA\(_f\) (14,540 cpm/pmol), salt components, and magnesium acetate as indicated. For the formation of eIF-2·[\(^{3}H\)]GDP, a mixture (75 μl) contained either 0.8 μg of Artemia eIF-2 or 0.57 μg of reticulocyte eIF-2, 0.63 μM \[^{3}H\]GDP (5,000–6,000 cpm/pmol), salt components, and magnesium acetate as indicated. \[^{35}S\]Met-tRNA\(_f\) binding to Artemia eIF-2 (○–○) and reticulocyte eIF-2 (△—△).

![Fig. 2. Reversal of Mg\(^{2+}\) inhibition of ternary complex formation by reticulocyte eIF-2. The standard ternary complex formation assay was used. The 75-μl reaction mixture contained 20 mM Tris-C1, pH 7.8; 100 mM KCl, 1 mM magnesium acetate, 0.2 mM GTP, 3 pmol of \[^{35}S\]Met-tRNA\(_f\) (11,350 cpm/pmol), 1 mM dithiothreitol, 75 μg of bovine serum albumin, 1.25 μg of reticulocyte eIF-2, and the nucleotide exchange factor as indicated. \[^{35}S\]Met-tRNA\(_f\) binding with (○—○) and without (□) magnesium acetate.

![Fig. 3. Exchange of unlabelled GTP with eIF-2-bound \[^{3}H\]GDP as a function of time. A typical 75-μl nucleotide exchange assay mixture contained 20 mM Tris-C1, pH 7.8, 100 mM KCl, 1 mM magnesium acetate, 75 μg of bovine serum albumin, 1 mM dithiothreitol, and approximately 0.5 pmol of eIF-2·[\(^{3}H\)]GDP complex. GTP (0.2 mM) and the nucleotide exchange factor (10 μg) were present as indicated. Nucleotide exchange with Artemia and reticulocyte binary complex are illustrated in A and B, respectively. No addition (○—○); 0.2 mM GTP (●—●); 0.2 mM GTP and 10 μg of nucleotide exchange factor (□—□).

![Fig. 4. Effect of Mg\(^{2+}\) on ternary and binary complex formation. Assay conditions were as described under "Experimental Procedures." For ternary complex formation, a 75-μl reaction mixture contained 0.8 μg of Artemia eIF-2 or 0.54 μg of reticulocyte eIF-2, 0.2 mM GTP, 3 pmol of \[^{35}S\]Met-tRNA\(_f\) (14,540 cpm/pmol), salt components, and magnesium acetate as indicated. For the formation of eIF-2·[\(^{3}H\)]GDP, a mixture (75 μl) contained either 0.8 μg of Artemia eIF-2 or 0.57 μg of reticulocyte eIF-2, 0.63 μM \[^{3}H\]GDP (5,000–6,000 cpm/pmol), salt components, and magnesium acetate as indicated. \[^{35}S\]Met-tRNA\(_f\) binding to Artemia eIF-2 (○—○) and reticulocyte eIF-2 (△—△).
which stimulates nucleotide exchange. Furthermore, with both eIF-2 preparations, formation of the ternary complex closely follows the loss of GDP from the binary complex.

A nonenzymic displacement on eIF-2-bound GDP was reported to occur in the reticulocyte system at concentrations of GTP greater than physiological (9). We have found that the concentration of free GTP used in nucleotide exchange with Artemia eIF-2-GDP complex does not affect the extent of nucleotide exchange (Table I). At a concentration of 8 μM (10 μM $K_r^{GTP}$), GTP exchanges with bound GDP, in the absence of any exchange promoting factor, as rapidly as at 200 μM.

### DISCUSSION

Formation of an eIF-2-GTP-Met-tRNA$_A$ ternary complex is followed by the transfer of this complex to a 40 S ribosomal subunit (5). When the 80 S initiation complex is formed upon addition of a 60 S subunit, mRNA, and other initiation factors, GTP hydrolysis occurs with a concomitant release of eIF-2 (5). Since the initiation factor is presumably released as a binary complex with GDP, and this binary complex is more stable ($K_r^{GDP} 3 \times 10^{-4}$ M) (20) than with GTP ($K_r^{GTP} 1 \times 10^{-6}$ M), it is important to understand how eIF-2 functions catalytically in vivo. Recently, Siekierka et al. (7) showed that in the absence of Mg$^{2+}$, GDP is exchangeable with exogenously added GTP to form eIF-2-GTP, the necessary precursor for ternary complex formation. In the presence of Mg$^{2+}$, GDP remains tightly bound to eIF-2 and prevents eIF-2 binding to GTP and Met-tRNA$'_A$.

We previously reported that Artemia eIF-2 binds GDP ($K_r^{GDP} 2.8 \times 10^{-8}$ M) to form a binary complex (12). In this communication, we report that this reaction is stimulated approximately 2-fold by the addition of 3-4 mM Mg$^{2+}$ (Fig. 1) and the bound GDP in the isolated binary complex exchanges for free GTP in the absence of any additional factor (Fig. 3A). We also confirm results of other laboratories (7, 16-18) that, in the presence of Mg$^{2+}$, eIF-2-GDP and eIF-2-GTP-Met-tRNA$'_A$ formation by reticulocyte eIF-2 are strongly inhibited (Figs. 1 and 2).

We have also partially purified a protein from the postribosomal supernatant or high salt wash of rabbit reticulocyte ribosomes. This protein is similar to several preparations that were shown to be high molecular weight protein complexes which may exist free or in combination with eIF-2 (7-10). The purified factor, in the presence of 1 mM Mg$^{2+}$, promotes the exchange of reticulocyte eIF-2 bound GDP with free GTP (Fig. 3B) and reverses the Mg$^{2+}$ inhibition of ternary complex formation (Fig. 2). However, this protein has little effect on the exchange of GDP for GTP with the Artemia eIF-2-GDP complex at 15 or 30 °C (Fig. 3A). As observed previously, the rate of exchange at 15 °C is slower than at 30 °C (11).

The addition of Met-tRNA$'_A$ does not appreciably change the rate of nucleotide exchange with the binary complex either from Artemia or rabbit reticulocytes (Figs. 3 and 4). The reticulocyte exchange factor, which has little effect on nucleotide exchange with the Artemia eIF-2-GDP complex, stimulates the Met-tRNA$'_A$ binding about 2.5-fold. Under similar conditions, Met-tRNA$'_A$ binding to reticulocyte eIF-2 is also stimulated approximately 4.5-fold by the factor. This is similar to previous reports from several laboratories (7-10).

Safer et al. (9) proposed that the requirement for a factor to promote GTP-GDP exchange, may be bypassed by increasing the GTP/GDP ratio. With excess GTP, the displacement of eIF-2-bound GDP by GTP would occur readily. We tested GTP at several concentrations in the nucleotide exchange and found that GTP exchanges freely in the absence of any exchange factor at its $K_r$ value as well as at 20 times the $K_r$ value (Table I).

Dormant embryos of Artemia contain a high concentration of GDP which decreases during development (21). The GTP/GDP ratio, which is 1.0 in dormant embryos, increases to 1.8 and 2.7 after 5.5 h and 36 h of development, respectively, suggesting that these nucleotides may have a role in regulating protein synthesis and development in Artemia embryos.

In summary, we have reported in this communication several significant differences between the Artemia and the reticulocyte system: (i) in the presence of Mg$^{2+}$, ternary and binary complex formation with Artemia eIF-2 are stimulated; (ii) Artemia eIF-2-GDP exchanges bound GDP readily with free GTP; (iii) in the presence of 1 mM Mg$^{2+}$, ternary and binary complex formation with reticulocyte eIF-2 are inhibited; and (iv) a protein factor isolated from reticulocytes is required for GDP-GTP exchange with reticulocyte eIF-2-GDP.

Whether a protein exists in brine shrimp embryos that accelerates recycling of eIF-2 in polypeptide chain initiation remains to be elucidated.

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REFERENCES