ASSOCIATION OF A GDP BINDING ACTIVITY WITH INITIATION FACTOR eIF-2 FROM CALF LIVER

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SUMMARY: A highly purified preparation of the eucaryotic initiation factor eIF-2 from calf liver which forms a ternary complex with GTP and Met-tRNA$_f^{Met}$ also exhibits a potent GDP binding activity. The factor preparation specifically forms a binary complex with GDP, other ribonucleoside diphosphates and GTP are inactive. Evidence is presented indicating that the GTP-dependent Met-tRNA$_f^{Met}$ binding and binary complex formation with GDP are mediated by the same protein which has an apparent molecular weight of 67,000 as judged by glycerol density gradient centrifugation.

INTRODUCTION: The initiation of protein synthesis in eucaryotic cells is a complex process requiring the participation of several protein factors (for a review, see ref. (1)). The exact number and identity of the factors as well as the details of their mechanism of action remain to be elucidated. One of the most widely studied factors is eIF-2[1/] which binds Met-tRNA$_f^{Met}$ in the presence of GTP to form a stable ternary complex Met-tRNA·eIF-2·GTP, which then binds to the 40S ribosomal subunit (1). Several laboratories have reported the isolation of a similar factor with the same general biological properties from a variety of eucaryotic sources. These include IF-E2(2), IF-MP(3) and EIF-1(4) from rabbit reticulocytes, IF-L3(5) from mouse L cells and IF-E3 from Krebs II ascites cells (6).

We have purified eIF-2 from the 0.5 M KCl ribosomal wash proteins from calf liver. During the course of purification of the factor we have observed that a GDP binding activity copurifies with the GTP-dependent Met-tRNA$_f^{Met}$ binding activity through a succession of DEAE-cellulose, hydroxylapatite and

1/ The factor nomenclature used here is based on the recent nomenclature agreement at the International Symposium on Protein Synthesis, October 18-20, 1976, National Institutes of Health, Bethesda, Md.

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phosphocellulose columns followed by glycerol gradient centrifugation. These results along with the observation that GTP-dependent Met-tRNA$_f^{Met}$ binding of eIF-2 is reversibly inhibited by GDP suggest that the GDP binding activity may be an intrinsic property of eIF-2.

MATERIALS AND METHODS

Purification of eIF-2 - The crude 0.5 M KCl ribosomal wash proteins were prepared from 600g of freshly thawed calf liver by a method similar to that of Picciano and Anderson (7). The eIF-2 activity in the 0.5 M KCl ribosomal wash proteins was removed by precipitation between 40% (226 g/l) and 60% (additional 151 g/l) ammonium sulfate saturation, then dissolved in 10 ml of Buffer A (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM methyl sulfonyl fluoride and 10% glycerol) containing 100 mM KCl. The solution was dialyzed for 10 hours against 1 liter of the same buffer, diluted in 1 volume of buffer A and applied to DEAE cellulose column (Whatman DE52, 2 x 30 cm) equilibrated in buffer A and 50 mM KCl. The column was washed successively with 200 ml volumes of buffer A containing (a) 50 mM KCl and (b) 100 mM KCl. The eIF-2 activity eluted in 100 mM KCl and fractions containing the bulk of the activity were pooled and adjusted to 50 mM potassium phosphate buffer, pH 7.5 and loaded onto a hydroxylapatite column (1 x 5 cm) equilibrated in buffer B (100 mM potassium phosphate buffer, pH 7.5, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM methylsulfonyl fluoride and 10% glycerol). The column was washed initially with 20 ml of buffer B followed by 20 ml of buffer B containing 300 mM potassium phosphate. The eIF-2 activity eluted in 300 mM potassium phosphate. The peak fractions of activity were pooled, diluted with 1 volume of 10% glycerol and applied to a column of phosphocellulose (0.5 x 2 cm). The adsorbed protein was eluted with a 16-ml linear gradient of 200 to 800 mM KCl in buffer A. A sharp peak of eIF-2 activity eluted in the region of the gradient containing 500 mM KCl. The active fractions were combined and dialyzed for 7 hours against buffer A containing 100 mM KCl and 50% glycerol. The final preparation (Fraction 5), assayed by the GTP-dependent Met-tRNA$_f^{Met}$ binding, bound 3750 pmol of Met-tRNA per mg of protein (see Table I). The binding reaction is specific for Met-tRNA$_f^{Met}$; [^{3}H]Phe-tRNA and [^{3}H]val-tRNA were tested in the binding reaction and were found to be inactive.

Other Materials - The purified rat liver tRNA$_f^{Met}$ used here was obtained from Biogenics Research Corporation and was charged with [^{35}S] methionine (6,000 cpm/pmol) by using an Escherichia coli synthetase preparation according to the method of Dubnoff and Maitra (8). The preparation contained approximately 1,000 pmol of methionine per A_{260} unit of t-RNA.

Assay for eIF-2 activity - Initiation factor activity was determined by measuring the ternary complex formation between eIF-2, Met-tRNA$_f^{Met}$ and GTP according to the procedure of Gupta et al (9). Reaction mixtures (75 µl) contained 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM 2-mercaptoethanol; 0.1 mM GTP, 0.01 A_{260} unit [^{35}S] Met-tRNA$_f^{Met}$ (containing 10 pmol of methionine) and eIF-2 (usually between 0.15 to 1.5 µg). After incubation for 3 min at 37°, the reaction mixtures were diluted with 3 ml of cold reaction buffer and filtered through nitrocellulose membrane filters which were then washed with 6 ml of cold reaction buffer, dried and counted in a liquid scintillation counter. One unit of eIF-2 activity was defined as the amount that promoted the GTP-dependent binding of 1 pmol of Met-tRNA$_f^{Met}$ to a nitrocellulose filter.

Assay of eIF-2-GDP complex formation - Reaction mixtures (75 µl) contained 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM 2-mercaptoethanol, 1.3 µM [^{3}H]GDP (2,000 cpm/pmol) and 0.15 to 1.5 µg of eIF-2. After incubation for 3 min at

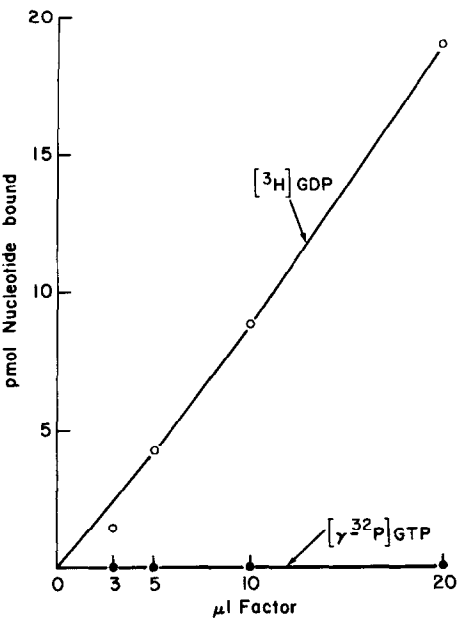


Fig. 1. Binding of guanine nucleotides to purified eIF-2. Binary complex formation was assayed as described under "Materials and Methods". Reaction mixtures contained 100 pmol of [^{3}H] GDP or [γ^{32}P] GTP and varying levels of eIF-2 as indicated.

37°, the reaction mixtures were diluted with 2 ml of cold reaction buffer and filtered through a nitrocellulose membrane filter. The filter was washed twice with 6 ml of the reaction buffer, dried and counted in a liquid scintillation counter. One unit of binding activity was defined as that amount of factor that promoted the binding of 1 pmol of GDP under the conditions of the assay described above.

RESULTS

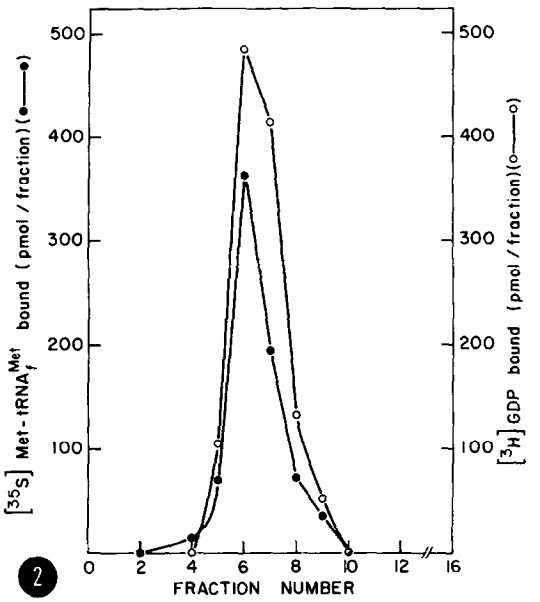
Binding of GDP to purified eIF-2 - We studied the interaction of guanine nucleotides with purified eIF-2 and found that addition of purified eIF-2 (phosphocellulose fraction) to [^{3}H]GDP in a reaction mixture containing no Met-tRNA$_f^{Met}$ resulted in the retention of radioactivity on nitrocellulose membrane filters. The amount of [^{3}H]GDP retained was proportional to the amount of eIF-2 added (Fig. 1). The binding reaction is specific for GDP. Replacement of GDP with GTP did not give binary complex formation (Fig. 1). Other ribonucleoside diphosphates were also tested in the binding reaction and were found to be inactive (data not shown).

TABLE I

Summary of Purification of eIF-2

Fraction	Total Units		Specific Activity		Ratio of ($^a/_b$)
	Met-tRNA Binding	GDP Binding	Met-tRNA Binding (a)	GDP Binding (b)	
			units/mg protein		
1. Crude KCl ribosomal wash	3,862	72,000	4.3	80	1:19
2. Ammonium sulfate	4,900	48,716	17	170	1:10
3. DEAE-Cellulose	1,140	8,300	51	368	1:7
4. Hydroxyl-apatite	984	1,862	82	158	1:1.8
5. Phospho-cellulose	525	1,136	3,750	8,114	1:2.1

Co-purification of GTP-dependent Met-tRNA$_f^{Met}$ binding and GDP binding activities - Table I summarizes the results of the purification of eIF-2. The protein fractions obtained at different stages of purification of eIF-2 were also assayed for GDP binding in the absence of Met-tRNA$_f^{Met}$. The results in Table I show that although large amounts of GDP binding activity unrelated to Met-tRNA$_f^{Met}$ binding were removed during purification, a significant amount copurified with Met-tRNA$_f^{Met}$ binding activity in a constant ratio of specific activities in the hydroxylapatite and the phosphocellulose fractions. Further evidence for the two activities residing in the same protein was provided by assaying successive fractions from the phosphocellulose column (the last purification step) for both activities (Fig. 2). Both Met-tRNA$_f^{Met}$ and GDP binding activities appeared in the same region of the chromatogram and the ratios of the two activities in the active fractions were identical, within the limits of the assay.



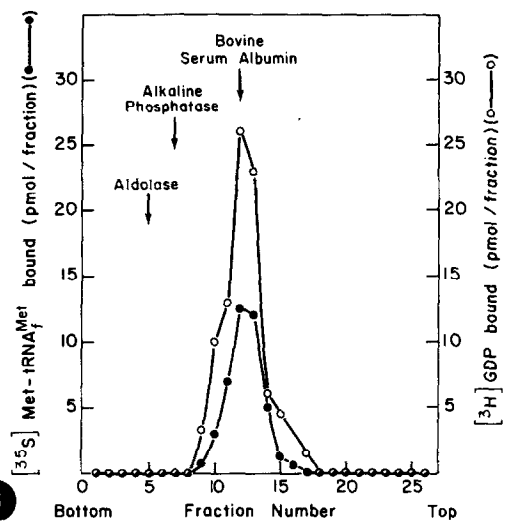


Fig. 2. Co-chromatography of Met-tRNA$_f^{Met}$ binding and GDP binding activities of eIF-2 on phosphocellulose. Fractions from phosphocellulose step of purification were assayed for Met-tRNA$_f^{Met}$ binding activity and GDP binding activity as described in "Materials and Methods".

Fig. 3. Glycerol gradient centrifugation of Met-tRNA$_f^{Met}$ binding and GDP binding activities of eIF-2. Purified eIF-2 (10 µg phosphocellulose fraction) was applied to a gradient of 10-30% glycerol (5.3 ml) in buffer A containing 100 mM KCl. Centrifugation was for 20 hr at 50,000 rpm at 5° in the SW 50.1 rotor. Fractions of 0.2 ml were collected and were assayed for Met-tRNA$_f^{Met}$ binding activity and GDP binding activity as described in "Materials and Methods".

Additional evidence that the Met-tRNA$_f^{Met}$ binding activity and GDP binding activity of eIF-2 are mediated by the same protein was obtained by glycerol density gradient centrifugation. Both activities cosedimented (Fig. 3), each active fraction again possessing the same ratio of the two activities.

This experiment also provided information about the native molecular weight of eIF-2, since marker proteins were run in parallel gradients. The sedimentation constant obtained for eIF-2 was 4.3 S. Assuming that eIF-2 is a typical globular protein ($\bar{v}$ = 0.73; f/fo = 1.25), the native molecular weight based upon sedimentation is approximately 67,000.

Inhibition Studies - Inhibition experiments were also employed to provide further evidence that GDP binding acvitity is an intrinsic property of eIF-2.

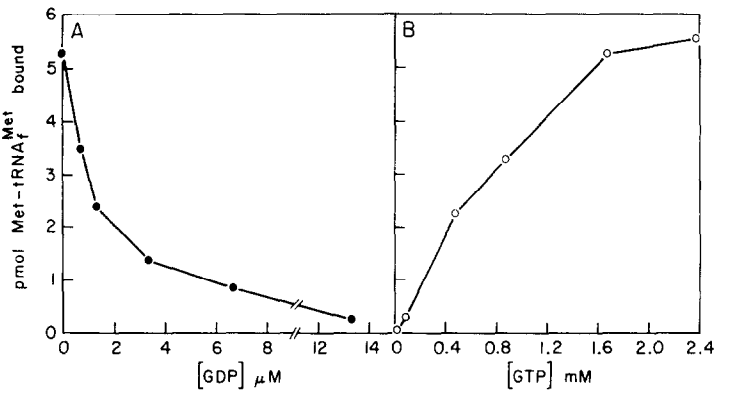


Fig. 4. GDP inhibition of eIF-2-mediated Met-tRNA$_f^{Met}$ binding. The conditions of GTP-dependent Met-tRNA$_f^{Met}$ binding were as described in "Materials and Methods". Reaction mixtures in experiment A contained 80 μM GTP and various levels of GDP as indicated. Each reaction mixture in experiment B contained 13 μM of GDP and various levels of GTP as indicated. The concentration of GDP used in experiment B caused virtually complete inhibition of Met-tRNA$_f^{Met}$ binding in the control reaction. All reactions were initiated by the addition of 1.5 μg of eIF-2 (phosphocellulose fraction).

GDP was found to be a potent inhibitor of GTP-dependent Met-tRNA$_f^{Met}$ binding by eIF-2 (Fig. 4A) and this inhibition was reversed by the addition of excess GTP (Fig. 4B). Inhibition of ternary complex formation by GDP has been observed by other workers with eIF-2 isolated from rabbit reticulocytes (10), wheat germ (11) and from A. Salina embryos (11).

DISCUSSION: Results presented in this communication demonstrate that purified eIF-2 preparations obtained from calf liver ribosomes exhibit a potent GDP binding activity as well as the ability to form a ternary complex with GTP and Met-tRNA$_f^{Met}$. This GDP binding activity is present in a constant ratio to the GTP-dependent Met-tRNA$_f^{Met}$ binding activity through the hydroxylapatite and phosphocellulose columns and glycerol density gradient centrifugation steps. These results together with those shown in Fig. 4 where GTP-dependent binding of Met-tRNA$_f^{Met}$ is inhibited by the addition of GDP and this inhibition is reversed by the addition of excess GTP, suggest that the GDP binding activity may be an intrinsic property of eIF-2. The significance of this activity in the overall mechanism of initiation of protein synthesis in eucaryotic cells

is unclear at the present time and is currently under investigation.

It is worth noting that the apparent native molecular weight of eIF-2 from calf liver is calculated to be approximately 67,000. This is in contrast to reported values of about 140,000 for eIF-2 purified from rabbit reticulocytes (6,13,14).

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