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Biochemistry

Rabbit reticulocyte initiation factor 2 contains two polypeptide chains of molecular weights 48,000 and 38,000

(eukaryotic initiation factor 2/Met-tRNA$_f^{Met}$ binding factor/ternary complex formation/40S ribosomal complex formation/ RNA binding to eukaryotic initiation factor 2)

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ABSTRACT **Eukaryotic initiation factor 2 (eIF-2) purified from rabbit reticulocyte lysates consists of equimolar amounts of two polypeptide chains of M_r 48,000 and 38,000. Determination of the molecular weight of the native factor gave a value which is consistent with a M_r of 86,000, indicating that the factor is composed of one M_r 48,000 and one M_r 38,000 polypeptide. The purified factor exhibited all the binding activities characteristic of eIF-2. The factor formed ternary complexes with Met-tRNA$_f^{Met}$ and GTP; it bound GDP to form a binary complex; and it also possessed the property of binding a wide variety of RNA species, including reoviral mRNA, phage T3 mRNA, rRNAs, and tRNA. Furthermore, the ternary complex formed by purified eIF-2 interacted with the 40S ribosomal subunit in the presence of AUG codon to form a 40S initiation complex. These results indicate that all binding activities attributed to eIF-2 are contained in the 48,000- and 38,000-dalton polypeptides.**

We have shown that eukaryotic initiation factor 2 (eIF-2) purified from ribosomal salt wash proteins from calf liver is composed of one 48,000- and one 38,000-dalton polypeptide corresponding to a molecular mass of 86,000 for the native factor (1). These results were in contrast to numerous reports that eIF-2 from other eukaryotic sources, and in particular from rabbit reticulocytes, consisted of three nonidentical polypeptide chains with molecular masses in the ranges 55,000–52,000, 50,000–48,000, and 38,000–35,000 daltons (2–11). In order to resolve the anomaly in the subunit composition (and molecular mass) of eIF-2 prepared from these two mammalian sources, we purified eIF-2 from rabbit reticulocyte lysates by the procedure used for preparation of the factor from calf liver.

In this paper we show that eIF-2 from rabbit reticulocytes that was purified by our rigorous purification procedure also produced only two polypeptide bands, of 48,000 and 38,000 daltons, on 15% polyacrylamide gels in the presence of sodium dodecyl sulfate. Similar to the eIF-2 from calf liver, our highly purified eIF-2 from rabbit reticulocytes exhibited all the activities known for eIF-2.

MATERIALS AND METHODS

Materials. [^{35}S]Met-tRNA$_f^{Met}$ was prepared by treating crude rabbit liver tRNA with [^{35}S]methionine (15,000 cpm/pmol) and an *Escherichia coli* synthetase preparation as described (1). Bacteriophage T3 [^{3}H]RNA was synthesized in a T3 DNA-directed T3 RNA polymerase reaction by using [^{3}H]UTP (20,000 cpm/nmol) as the labeled substrate (12). Reovirus cores, prepared from purified virus by chymotrypsin digestion, were the gift of Aaron J. Shatkin (Roche Institute of Molecular Biology). These washed viral cores were used to direct the synthesis of methylated "capped" viral [^{3}H]mRNA by using [^{3}H]GTP (30,000 cpm/nmol) as the labeled substrate, and subsequent isolation of the viral RNA from polymerase reactions was accomplished as described (13). Ribosomal subunits were prepared from *Artemia salina* embryos by the procedure of Zasloff and Ochoa (14).

Assay of eIF-2 Activity. The assay of Met-tRNA$_f^{Met}$ binding to eIF-2 by using nitrocellulose filtration followed the procedure of Gupta *et al.* (15) as outlined (1). Reaction mixtures (75 μl) contained 20 mM Tris·HCl (pH 7.5), 100 mM KCl, 2 mM 2-mercaptoethanol, 0.2 mM GTP, 0.25–0.5 A_{260} unit of [^{35}S]Met-tRNA$_f^{Met}$ (containing 5–10 pmol of methionine), and eIF-2. Incubation was at 37°C for 3 min. One unit of eIF-2 activity was defined as the amount that promoted the binding of 1 pmol of Met-tRNA$_f$ to a nitrocellulose membrane filter.

Preparation of Crude Ribosomal Salt Wash Proteins from Rabbit Reticulocyte Lysates. All operations were carried out at 0–4°C unless otherwise indicated. A 600-ml sample of frozen rabbit reticulocyte lysates (GIBCO) was diluted with an equal volume of buffer (0.25 M sucrose/20 mM Tris·HCl, pH 7.5/35 mM KCl/10 mM $MgCl_2$/0.1 mM EDTA/1 mM dithiothreitol). The diluted lysate was centrifuged in a Sorvall centrifuge at 4000 rpm for 10 min and at 8000 rpm for 20 min. The supernatant material was filtered through glass wool and then centrifuged for 3 hr at 55,000 rpm in a Spinco rotor 60 Ti. The crude ribosomal pellets were suspended in 50 ml of buffer (0.25 M sucrose/50 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/5 mM $MgCl_2$/0.1 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride) and treated with 2 μg of pancreatic DNase per ml for 20 min at 20°C. The suspension was made up to 0.5 M KCl by the slow addition of 4 M KCl, was stirred for 30 min at 4°C, and then was centrifuged for 3 hr at 55,000 rpm in a Beckman 60 Ti rotor. The supernatant material was dialyzed overnight against 2 liters of buffer A [50 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/0.1 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/10% glycerol (vol/vol)]. Following dialysis, the ribosomal wash fluid was fractionated by adding solid ammonium sulfate (361 g/liter). The precipitated material was dissolved in 20 ml of buffer A containing 100 mM KCl and was dialyzed for 10 hr against 1 liter of the same buffer.

The DNase treatment followed by fractionation with ammonium sulfate removed nucleic acids and many of the proteins associated with DNA from the crude eIF-2 preparation. These procedures resulted in starting material with a relatively high specific activity as shown by fraction I in Table 1.

Purification of eIF-2. Further purification steps were carried out between 0 and 4°C except where indicated. All buffer solutions contained 1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM

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Abbreviation: eIF-2, eukaryotic initiation factor 2.

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Table 1. Purification of eIF-2

Fraction	Total units	Total proteins, mg	Specific activity units/mg protein*
Ammonium sulfate I	11,220	66	170
Ammonium sulfate II	6,000	20	300
DEAE-Cellulose	3,300	3	1100
CM-Sephadex	2,400	0.95	2526
Bio-Rex 70	1,100	0.25	4400
Sucrose gradient	500	0.08	6250

* Protein was determined by the Bio-Rad assay method of Bradford (16).

phenylmethylsulfonyl fluoride, and 10% glycerol (vol/vol); all glassware was siliconized before use. The crude initiation factor (fraction I; 20 ml containing 65 mg of protein obtained from 600 ml of lysate) was diluted with an equal volume of buffer A containing 0.4 M KCl. Ten grams (wet weight) of DEAE-cellulose (DE-52), previously equilibrated with buffer A containing 0.25 M KCl, was added to the diluted fraction I. The suspension was stirred for 2 hr, poured into a small column, and allowed to settle. The protein containing all the eIF-2 activity was eluted from the column by washing with buffer A containing 0.25 M KCl. The eluted protein fractions were pooled and fractionated by adding solid ammonium sulfate. The material precipitating between 40% ammonium sulfate (226 g/liter) and 60% (346 g/liter) was dissolved in 10 ml of 20 mM Tris·HCl (pH 7.5)/100 mM KCl and was dialyzed overnight against 2 liters of this buffer.

These procedures yielded the ammonium sulfate fraction II (10 ml containing 20 mg of protein), which was diluted with an equal volume of 20 mM Tris·HCl, pH 7.5, to give a final KCl concentration of 50 mM. The solution was loaded onto a 5-ml DEAE-cellulose column that had been equilibrated with 25 mM Tris·HCl (pH 7.5)/50 mM KCl; the column was washed with this buffer until A_{280} was less than 0.1. A linear gradient (30-ml total volume) from 25 mM Tris·HCl (pH 7.5)/50 mM KCl to 25 mM Tris·HCl (pH 7.5)/300 mM KCl was applied to the column, 0.5 ml fractions were collected, and eIF-2 was eluted between 150 and 200 mM KCl.

The fractions containing the bulk of the eIF-2 activity were pooled (DEAE-cellulose eluate, fraction III). The DEAE-cellulose eluate (10 ml containing 3 mg of protein) was adjusted to pH 7.3 by the addition of 0.5 M KH_2PO_4 and was applied to a CM-Sephadex C-50 column (3-ml bed volume) that had been equilibrated in 20 mM potassium phosphate (pH 7.3)/100 mM KCl. After slow loading of the sample, the column was washed with 20 ml of 20 mM potassium phosphate (pH 7.3)/150 mM KCl. A linear gradient (24-ml total volume) from 20 mM potassium phosphate (pH 7.3)/150 mM KCl to 20 mM potassium phosphate (pH 7.3)/800 mM KCl was applied to the column, and the eIF-2 activity was eluted between 300 and 350 mM KCl. The fractions containing the eIF-2 activity were pooled (CM-Sephadex eluate, fraction IV).

This CM-Sephadex eluate (approximately 10 ml) was adjusted to pH 6.5, diluted with an equal volume of 20 mM potassium imidazole (pH 6.5), and then applied to a column (0.5-ml bed volume) of Bio-Rex 70 which had been equilibrated in 20 mM potassium imidazole, pH 6.5/150 mM KCl. The column was washed with 5 ml of 20 mM potassium imidazole, pH 6.5/150 mM KCl and then with two additional 5-ml washes with 20 mM potassium imidazole at (pH 6.5), the first containing 300 mM and the second 600 mM KCl in 7% glycerol (vol/vol) and no EDTA. Fractions (0.25 ml) were collected; all eIF-2 activity was eluted with 600 mM KCl (Bio-Rex-70 eluate, fraction V). Approximately 0.3-ml fractions of the Bio-Rex-70 eluate were layered onto 10% to 30% (wt/vol) sucrose gradients (12-ml total volume) in 20 mM potassium phosphate, pH 7.0/150 mM KCl in centrifuge tubes that were placed in a Spinco SW 41 rotor, and the gradients were centrifuged at 40,000 rpm for 48 hr. Fractions (0.25 ml) were collected from the bottom of the tubes, and the peak fractions of eIF-2 activity were pooled and stored in small aliquots at −70°C in a Revco unit until use (fraction VI). The results of a typical purification of eIF-2 are presented in Table 1.

Because purification of eIF-2 from rabbit reticulocytes requires much smaller quantities of starting material than in the case of calf liver, the time of purification is relatively shorter and the proportion of eIF-2 inactivated is much less. Hence, the specific activity of fraction VI eIF-2 is almost double that from calf liver. The overall yield was almost 5%, which is similar to values reported previously (1, 17).

RESULTS

Subunit Composition and Molecular Weight. Purified eIF-2 (fraction VI) was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis on gels containing 15% acrylamide (Fig. 1). It is evident that fraction VI exhibited only two polypeptide bands with molecular weights of 48,000 and 38,000. When fraction VI was run in parallel with highly purified calf liver eIF-2, the two polypeptide bands migrated identically and no differences in molecular weight could be detected (data not shown).

Densitometric tracings of gels showed that fraction VI possessed the 48,000- and 38,000-dalton polypeptide chains in a 1:1 molar ratio (Fig. 2). These values are consistent with those reported for calf liver eIF-2 but are in contrast to published data in which similar quantities of protein showed three distinct bands in approximately equimolar ratios (2, 5).

The apparent native molecular weight of eIF-2 was calculated by determining (*i*) the sedimentation coefficient of eIF-2 by glycerol density gradient centrifugation (Fig. 3) and (*ii*) the Stokes' radius by Sephadex G-200 gel filtration under conditions described (1) for calf liver eIF-2 (data not shown). From these experiments, it was calculated by the methods of Martin and Ames (18) and by Ackers (19) that rabbit reticulocyte eIF-2 has a sedimentation coefficient of 5.2 S and a Stokes' radius of 36

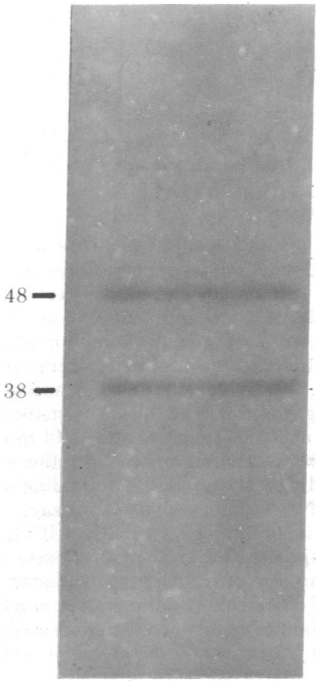

FIG. 1. Sodium dodecyl sulfate/polyacrylamide gel electropherograms of eIF-2 preparations. Purified eIF-2 (fraction VI, 8 μg) was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis in gels containing 15% polyacrylamide by the method of Schreier *et al.* (2) as described (1). A set of marker proteins of known molecular weight (shown × 10^{-3}) were run in parallel lanes on the same gel (not shown).

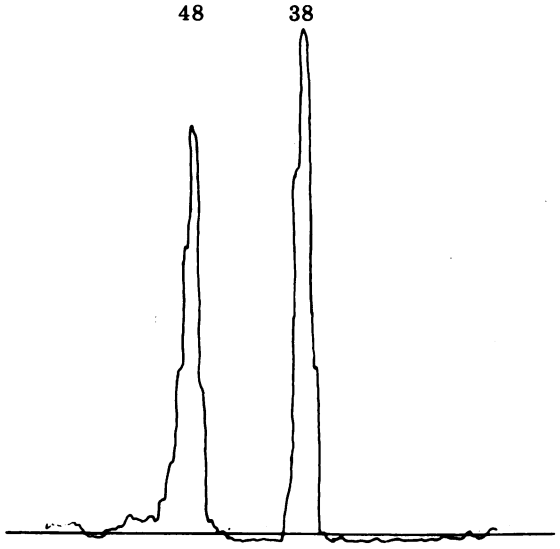

FIG. 2. Densitometric tracings of purified eIF-2. The stained gel of Fig. 1 was scanned at 540 nM in a Gilford gel scanner attached to a Zeiss PM6 spectrometer. Molecular weights are shown × 10^{-3}.

Å. Calculation of native molecular weight of eIF-2 from these data by the method of Siegel and Monty (20) gave a value of 77,000. This value is consistent with a native molecular weight of 86,000 resulting from one subunit of 48,000 and one of 38,000 as determined by sodium dodecyl sulfate/gel electrophoresis.

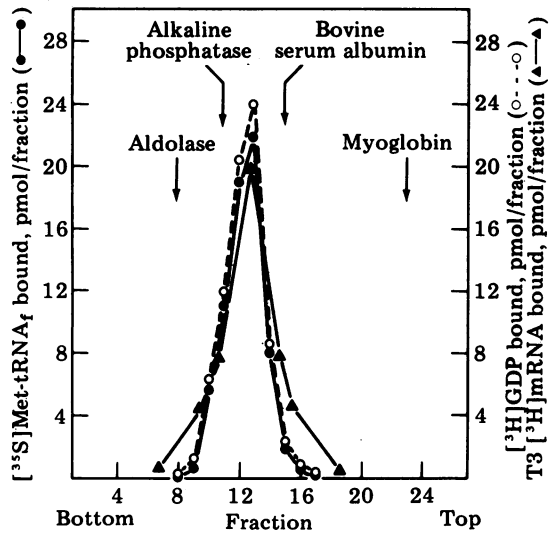

FIG. 3. Glycerol gradient sedimentation of eIF-2. Purified eIF-2 (fraction VI, 80 units) was mixed with 200 μg of crystalline rabbit muscle aldolase, 1 mg of bovine serum albumin, and 45 μg of beef heart catalase in a total volume of 0.1 ml and layered onto a 10–30% glycerol (wt/vol) linear gradient (5.2 ml) containing 20 mM potassium phosphate (pH 7.0), 0.15 M KCl, and 1 mM dithiothreitol. A marker gradient containing catalase (45 μg), muscle aldolase (200 μg), and bacterial alkaline phosphatase (0.5 mg) was run in parallel. The gradients were centrifuged for 20 hr at 4°C at 50,000 rpm in the SW 50.1 rotor. Fractions (0.2 ml) were collected from each tube and each fraction was assayed for [^{35}S]Met-tRNA$_f$ binding activity. [^{3}H]GDP binding was assayed as described (17), and RNA binding activity was assayed as follows. Reaction mixtures (75 μl) containing 20 mM Tris·HCl (pH 7.5), 150 mM KCl, 2 mM 2-mercaptoethanol, and 5 pmol of bacteriophage T3 [^{3}H]RNA (20,000 cpm/pmol of RNA) were incubated at 37°C for 3 min, diluted with 3 ml of ice-cold reaction buffer, and filtered through nitrocellulose membrane filters which then were washed with 6 ml of reaction buffer. The filters were dried and assayed for radioactivity in a liquid scintillation counter.

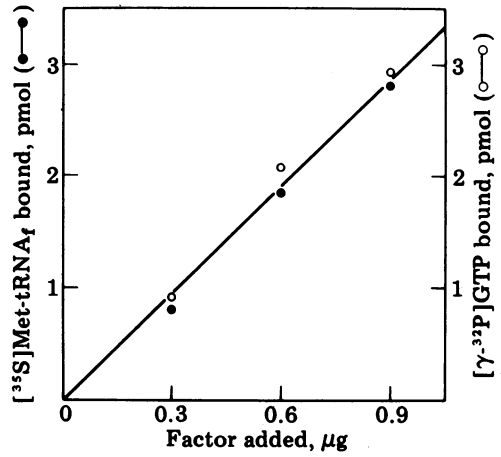

FIG. 4. Binding of Met-tRNA$_f$ and GTP to reticulocyte-eIF-2. Ternary complex formation was measured as described except that reaction mixtures (50 μl) contained 5 μg of dialyzed bovine serum albumin, 8 μM [γ-^{32}P]GTP (4000 cpm/pmol) or [^{35}S]Met-tRNA$_f$ (containing 10 pmol of methionine, 20,000 cpm/pmol), and varying levels of eIF-2 as indicated. After incubation for 3 min at 37°C, reaction mixtures were diluted with 2 ml of reaction buffers and filtered through membrane filters that had been prewashed with reaction buffers containing 25 μM unlabeled GTP. In the absence of eIF-2, the amount of ^{35}S and ^{32}P bound was <0.01 and <0.2 pmol, respectively. It should be noted that, under the above suboptimal assay conditions, the Met-tRNA$_f$ binding activity of eIF-2 was reduced to 60% of the value obtained in standard assay mixtures.

Properties of Purified Reticulocyte eIF-2 Containing Two Subunits. Purified reticulocyte eIF-2 possesses all the activities that have been attributed to this initiation factor. The factor bound Met-tRNA$_f$ and GTP in a 1:1 molar ratio (Fig. 4). Purified eIF-2 also bound GDP to form a binary complex (Fig. 3), a property characteristic of purified eIF-2 preparations (17, 21). The molar ratio of Met-tRNA$_f$ binding to GDP binding was close to 1:1 (Fig. 3). In addition, in agreement with the reports published from a number of laboratories (22, 23), the purified factor also possessed an RNA binding activity (Fig. 3). As with the GDP binding activity, RNA binding activity coincided with the Met-tRNA$_f$ binding activity of eIF-2. The RNA binding activity of eIF-2 is not restricted to bacteriophage mRNAs. In addition to T3 mRNA, other RNA preparations, *E. coli* [^{32}P]-rRNAs, and reovirus [^{3}H]mRNA also bound eIF-2 with nearly equal facility (Fig. 5). At a relatively higher concentration (100 pmol/0.1 ml), *E. coli* [^{32}P]tRNA also bound to purified eIF-2 preparation.

The ternary complex formed by purified eIF-2 efficiently transferred Met-tRNA$_f$ to 40S ribosomes in the presence of AUG

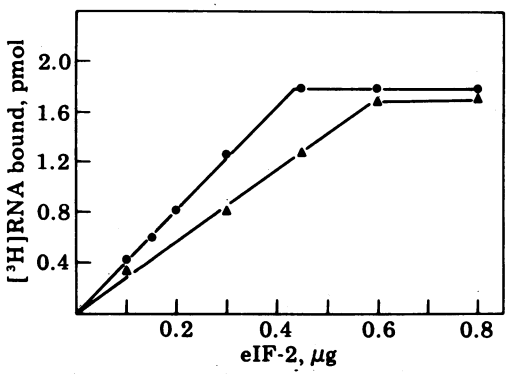

FIG. 5. Binding of RNA to purified eIF-2. Reaction mixtures were prepared as described in the legend to Fig. 3 except that 3 pmol of either methylated "capped" reovirus [^{3}H]mRNA (15,000 cpm/pmol of RNA) or *E. coli* 23S and 16S [^{32}P]rRNAs (25,000 cpm/pmol) were added. ●, Reovirus [^{3}H]mRNA; ▲, [^{32}P]rRNAs.

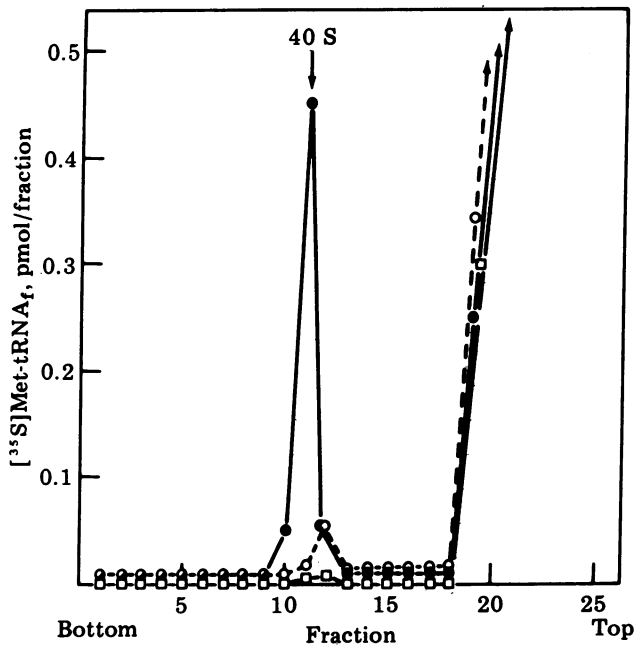

FIG. 6. Formation of the 40S initiation complex. The reaction was carried out in two stages. The first stage involved formation of an eIF-2–GTP–[^{35}S]Met-tRNA$_f$ ternary complex; the second stage involved transfer of the ternary complex to 40S ribosomal subunits. The components in stage I incubation (total volume, 0.15 ml) were as described except that 1.8 units of purified eIF-2 (fraction VI) was used. After incubation at 37°C for 3 min to form the ternary complex, $MgCl_2$ was added to the reaction mixtures to a concentration of 5 mM, followed by 0.1 A_{260} unit of AUG codon and 0.26 A_{260} unit of 40S ribosomal subunits. After incubation for 3 min at 37°C, the reaction mixtures were chilled, and 0.1 ml was layered onto a 5-ml linear 5–20% (wt/vol) sucrose gradient containing 20 mM Tris·HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, and 2 mM 2-mercaptoethanol and centrifuged for 90 min at 50,000 rpm at 4°C in a Spinco SW 50.1 rotor. Fractions (0.2 ml) were collected and ^{35}S radioactivity was measured in Aquasol (New England Nuclear). ●, Complete system; ○, without AUG; □, without eIF-2.

codon, and the ribosomal binding reaction was dependent on both AUG codon and eIF-2 (Fig. 6). The dependence on AUG codon for 40S initiation complex formation was first demonstrated by Gupta *et al.* (15).

DISCUSSION

Results presented in this paper demonstrate that eIF-2 purified from rabbit reticulocyte lysates contains two polypeptide chains, of molecular weights 48,000 and 38,000, in a 1:1 ratio. When rabbit reticulocyte and calf liver eIF-2 preparations were run in parallel in the gel system, the corresponding polypeptides of eIF-2 from each source had identical mobilities. These results together with similar values of native molecular weight of eIF-2 purified from both sources lead us to conclude that, in the native state, eIF-2 purified from rabbit reticulocyte lysates consists of two polypeptide chains of molecular weights 48,000 and 38,000.

Barrieux and Rosenfeld have shown that the Met-tRNA$_f^{Met}$ binding activity resides on the 48,000-dalton polypeptide and the GDP binding activity resides on the 38,000-dalton polypeptide of rabbit reticulocyte eIF-2 (7). Since each activity resides on a different polypeptide, purified factors containing the polypeptides in a 1:1 molar ratio would also be expected to exhibit GDP binding and Met-tRNA$_f^{Met}$ binding activities in a similar ratio. We have shown this to be true with our eIF-2 preparation containing the two polypeptide chains. After glycerol density gradient centrifugation, the GDP and Met-tRNA$_f^{Met}$ binding activities of eIF-2 were coincident and the molar ratios of GDP and Met-tRNA$_f^{Met}$ bound were 1:1. In addition to Met-tRNA$_f$ and GDP binding activities, the purified factor possesses all other binding activities attributed to eIF-2, including its ability to promote transfer of Met-tRNA$_f$ to 40S ribosomes in the presence of AUG codon.

These results allow us to conclude that the pure eIF-2 is composed of one 48,000- and one 38,000-M_r polypeptide, corresponding to a M_r = 86,000 for the native factor. The isolation of such a factor from two different sources—namely, rabbit reticulocytes and calf liver—indicates that the appearance of a two-polypeptide-chain protein factor is not a phenomenon restricted to calf liver alone. This is a significant finding because of the central role played by eIF-2 in initiation complex formation and its involvement in translational control through its interaction with the protein kinase activities of the heme-controlled repressor and with those induced by double-stranded RNA and interferon (see ref. 11 for review).

We conclude that the third polypeptide band that other workers have found in their preparations purified from rabbit reticulocytes is not an intrinsic component of eIF-2, although the possibility that it may play a role in subsequent steps of initiation complex formation cannot yet be ruled out.

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