Purification and Characterization of Eukaryotic Initiation Factor 2 and a Guanine Nucleotide Exchange Factor from Rat Liver*

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Two polypeptide chain initiation factors, eukaryotic initiation factor 2 (eIF-2) and guanine nucleotide exchange factor (GEF), were isolated from rat liver. Two forms of eIF-2 were identified, one contained three subunits (α, β, and γ), and the other contained only the α- and γ-subunits. The three-subunit form was similar to eIF-2 from rabbit reticulocytes with respect to the sedimentation coefficient, Stokes radius, molecular weight of the α- and γ-subunits, ability to restore protein synthesis in hemin-deficient reticulocyte lysate, and immunological cross-reactivity of the α-subunits using antibodies against liver eIF-2. In contrast, the β-subunits of the liver and reticulocyte factors were distinct; they had different molecular weights, and antibodies against rat liver eIF-2β did not recognize the β-subunit of the reticulocyte factor. Furthermore, the GDP dissociation constant for reticulocyte eIF-2 was more than twice that of the liver factor. GEF from rat liver reversed GDP inhibition of the ternary complex assay and catalyzed the exchange of eIF-2-bound GDP for free GDP or GTP, characteristics ascribed to the corresponding protein from rabbit reticulocytes. However, its subunit composition and molecular weight were different from those reported for reticulocyte GEF. The $T_{m}$ for GDP exchange mediated by GEF was about 5-fold slower with two-subunit than with three-subunit eIF-2. In addition, the $K_{D}$ for GDP was lower for two-subunit than for three-subunit eIF-2 when GEF was present. Taken together, these data demonstrate species-associated variability in the β-subunit of eIF-2 and suggest a crucial role for the β-subunit in the functional interaction of eIF-2 and GEF.

The transfer of initiator Met-tRNA and GTP to the 40 S ribosomal subunit is mediated through eukaryotic initiation factor 2 (eIF-2β) (1). The 40 S preinitiation complex can then bind to mRNA, and a 60 S ribosomal subunit to form a complete 80 S initiation complex. Formation of the ternary complex between eIF-2, GTP, and Met-tRNA, in rabbit reticulocytes is controlled through the interaction of eIF-2 with a guanine nucleotide exchange factor (GEF). GEF catalyzes the exchange of GDP bound to eIF-2 for free GTP (2-4). Since GDP association with eIF-2 inhibits the binding of Met-tRNA, GEF-mediated exchange of GTP for GDP in the eIF-2-GDP binary complex is a prerequisite for maximum ternary complex formation.

GEF isolated from a number of sources including rabbit reticulocytes (5), rabbit skeletal muscle (6), HeLa cells (7), and Ehrlich ascites cells (8) consists of three dissimilar subunits. In contrast, the subunit composition of eIF-2 from liver is less well defined; one report describes the isolation of a two-subunit factor (9), whereas another report suggests the presence of a three-subunit factor (10, 11). The majority of the physicochemical characterization of eIF-2 has been done using three-subunit factor isolated from rabbit reticulocytes (5, 12). The only physical characterization of eIF-2 from liver, other than determination of subunit composition and molecular weight, has been performed using factor containing only two subunits (9). Also, only a single report has been published comparing the immunological cross-reactivity of rabbit reticulocyte eIF-2β subunits with the eIF-2β subunits from another source (HeLa cells) (13).

GEF has been purified from rabbit reticulocytes (2), Ehrlich ascites tumor cells (3), and HeLa cells (14). GEF from rabbit reticulocytes consists of five dissimilar subunits of $M_r$, 26, 39, 58, 67, and $82 \times 10^3$. The exact molecular weights of the subunits of GEF from HeLa cells have not been reported. However, a comparison of the electrophoretic mobility of the subunits of the protein from HeLa cells to that of rabbit reticulocytes shows that, whereas both proteins have five subunits, they only have one subunit with the same molecular weight (58,000). GEF from Ehrlich cells consists of six subunits of $M_r$, 21, 27, 37, 52, 67, and $85 \times 10^3$. Only three of the subunits of GEF from Ehrlich cells have the same molecular weight as the reticulocyte protein (i.e. 26, 39, and 67 × 10³). GEF isolated from Ehrlich cells functions in an analogous manner to the reticulocyte factor in in vitro assays, but does not reverse the inhibition of protein synthesis in hemin-deficient reticulocyte lysate (3). This, together with the observation that GEF from rabbit reticulocytes has little effect on the guanine nucleotide exchange of eIF-2 from Artemia embryos (15), indicates that GEF from various sources may have different physical and biological properties.

In the present report, we describe the purification of both two- and three-subunit eIF-2, as well as GEF from rat liver. We also compare the physicochemical, biological, and immunological characteristics of liver eIF-2 to the rabbit reticulocyte factor and characterize liver GEF.
EXPERIMENTAL PROCEDURES

RESULTS

Purification of eIF-2 from Rat Liver—A summary of the purification of eIF-2 from rat liver using conventional methodology, as well as FPLC, is shown in Table I. Activity of eIF-2 could not be determined accurately in steps prior to ammonium sulfate precipitation. A similar problem was observed during purification of eIF-2 from rabbit skeletal muscle and was attributed to the presence of nondialyzable factors such as unlabeled Met-tRNA, which would interfere with the binding of [35S]Met-tRNA in the eIF-2 assay. The specific activity of Step 5a eIF-2 was 25% of that reported for eIF-2 from rabbit skeletal muscle (6), two-subunit eIF-2 from rat liver (9), or three-subunit eIF-2 from rat liver (Table I, Step 4b). This was due to the presence of 0.2 M Mg2+ and nonsaturating concentrations of Met-tRNA in the assay. Assay of the Step 5a factor with 0.2 M Mg2+ and saturating Met-tRNA gave a specific activity of 3570 pmol/mg, which was comparable to that observed with other purified preparations of eIF-2.

The first two steps of the conventional and FPLC purification schemes were identical. In the third step of the FPLC procedure, eIF-2 was chromatographed on a Mono Q anion exchange column (Table I, Step 3b). At this step, eIF-2 was separated into two peaks of activity; one contained three-subunit eIF-2 which eluted at 230 mM KCI, and the other contained eIF-2 which lacked the β-subunit and eluted at 285 mM KCI. The ratio of two- to three-subunit eIF-2 varied from one preparation to another, but was generally less than 0.2. The samples were diluted to adjust the KCI concentration to approximately 60 mM and immediately applied to a Mono S cation exchange column (Table I, Step 4b). Three-subunit eIF-2 eluted from this column at 250 mM KCI, whereas the two-subunit factor eluted at 290 mM KCI. A major advantage of the FPLC procedure over the conventional purification scheme was that it took only 5 days to complete rather than 11 days.

Characterization of Rat Liver eIF-2—Conditions used to determine physical properties of the eIF-2 holoprotein (Table II) were chosen to prevent aggregation of the protein, i.e. the protein concentration was kept below 0.75 mg/ml, and the KCI concentration was 0.7 M in all buffers. Under these conditions, the apparent molecular weight of eIF-2 determined by gel filtration chromatography on Sephacryl S-200 was similar to that calculated from the Stokes radius and sedimentation coefficient. In contrast, the molecular weight calculated by SDS-polyacrylamide gel electrophoresis was much lower than that found using either of the other two methods. The discrepancy between the molecular weight determined by SDS-polyacrylamide gel electrophoresis and that determined by the other two methods is most easily explained by a deviation from sphericity of the holoprotein. Assuming that eIF-2 is a prolate ellipsoid, an axial ratio of 6.7 was calculated from a plot of (-log Ks) vs. Ks, where Ks is the sedimentation coefficient. The sedimentation coefficient of eIF-2 was determined by its rate of sedimentation in a 10–30% sucrose gradient (SW-60 rotor, 54,000 rpm for 20 h) compared to the rate observed for proteins of known sedimentation coefficient (cytochrome c, chymotrypsinogen, ovalbumin, bovine serum albumin, and aldolase). The molecular weight of rat liver eIF-2 was calculated from the sedimentation coefficient and Stokes radius. The molecular weight of eIF-2 subunits was determined by SDS-polyacrylamide gel electrophoresis (PAGE) as described in Fig. 1. The pl of eIF-2 subunits was determined by two-dimensional electrophoresis. Isoelectric focusing gels contained 0.5 M urea, 2% Nonidet P-40, and 2% pH 4–5 ampholytes and were focused for 12,000 V-h.  

<table>
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<tr>
<th>Step</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
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<td>1a.</td>
<td>40–52% ammonium sulfate</td>
<td>2582.8</td>
<td>35,000</td>
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<td>2a.</td>
<td>Phosphocellulose</td>
<td>66.6</td>
<td>17,600</td>
</tr>
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<td>3a.</td>
<td>DEAE-cellulose</td>
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<td>4a.</td>
<td>Sephacryl S-200</td>
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<td>10,200</td>
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<td>5a.</td>
<td>10–30% sucrose gradients</td>
<td>5.7</td>
<td>4,700</td>
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<tr>
<td>1b.</td>
<td>40–52% ammonium sulfate</td>
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<td>9,760</td>
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<tr>
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<td>FPLC Mono Q</td>
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<td>FPLC Mono S</td>
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Physical properties of eIF-2

The physical properties of rat liver eIF-2 from Step 5a (Table I) were determined by gel filtration chromatography and sucrose gradient centrifugation as described by Sherman (27). eIF-2 (0.2 ml, 0.7 mg/ml) was gel-filtered at a flow rate of 7 ml/h on a 0.9 × 30-cm Sephacryl S-200 column in Buffer C containing 0.7 M KCl. The apparent molecular weight of the factor was calculated from a plot of log(molecular weight) versus elution volume using the following proteins as standards: aldolase, ovalbumin, and cytochrome c. The Stokes radius of eIF-2 was calculated from a plot of (-log Ks) vs. Ks. The sedimentation coefficient of eIF-2 was determined by its rate of sedimentation in a 10–30% sucrose gradient (SW-60 rotor, 54,000 rpm for 20 h) compared to the rate observed for proteins of known sedimentation coefficient (cytochrome c, chymotrypsinogen, ovalbumin, bovine serum albumin, and aldolase). The molecular weight of rat liver eIF-2 was calculated from the sedimentation coefficient and Stokes radius. The molecular weight of eIF-2 subunits was determined by SDS-polyacrylamide gel electrophoresis (PAGE) as described in Fig. 1. The pl of eIF-2 subunits was determined by two-dimensional electrophoresis. Isoelectric focusing gels contained 0.5 M urea, 2% Nonidet P-40, and 2% pH 4–5 ampholytes and were focused for 12,000 V-h.

Table II

Physical properties of eIF-2

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<td>10,700</td>
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<td>4a.</td>
<td>Sephacryl S-200</td>
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<tr>
<td>5a.</td>
<td>10–30% sucrose gradients</td>
<td>5.7</td>
<td>4,700</td>
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Molecular weight

Gel filtration

Stokes radius and sedimentation coefficient

SDS-PAGE

Sedimentation coefficient (S)

Stokes radius (A)

Friction coefficient

Liver

Reticulocyte

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<td>γ</td>
<td>48,300</td>
<td>52,000</td>
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*From Safer et al. (12).

#From Lloyd et al. (5).
contained a low concentration of bisacrylamide (0.08%) because higher concentrations of the cross-linker (0.2%) caused the β- and γ-subunits to co-migrate. The relative molecular weights of rat liver eIF-2 subunits (α, β, and γ) under these conditions were 34,000, 54,800, and 48,300 (Table II). As seen in Fig. 1, the α- and γ-subunits of eIF-2 from rat liver and rabbit reticulocytes migrated identically. However, the β-subunit of liver eIF-2 electrophoresed as a single band during SDS-polyacrylamide gel electrophoresis, while the reticulocyte β-subunit electrophoresed as an incompletely separated doublet.

The isoelectric points of the α- and β-subunits of rat liver eIF-2, as determined by two-dimensional gel electrophoresis (Table II), were in good agreement with those reported for the reticulocyte subunits (5, 20). However, the isoelectric point of the γ-subunit of rat liver eIF-2 could not be accurately determined, since under these conditions it focused as a broad band around pH 8.5.

At either the Sephacryl S-200 chromatography step or the Mono Q step of the purification procedure, rat liver eIF-2 was separated into two species; one contained a protein exhibiting the three subunits typical of eIF-2 from other sources, whereas the other contained only the α- and γ-subunits (Fig. 1). The identity of the missing subunit was confirmed by two-dimensional gel electrophoresis. The β-subunit from eIF-2 containing all three subunits had the expected molecular weight and isoelectric point (54,800 and 5.8, respectively). In contrast, this subunit was completely absent in the two-subunit eIF-2 sample. The two-subunit form of eIF-2 also contained in some preparations a Mr = 17,000 protein present in approximately proportional amounts to the α- and γ-subunits.

Antibodies raised in rabbits against rat liver eIF-2 recognized the protein from both liver and reticulocytes. However, the reaction with reticulocyte eIF-2 was less sensitive than that observed for the liver factor (Fig. 2a). The basis for this difference in sensitivity can be seen in Fig. 2. Whereas the antibody recognized the α-subunit of both liver and reticulocyte eIF-2, the β-subunit of only the liver factor showed reactivity with the antibody. The relative mobility of the β-subunit of purified eIF-2 was the same as that of eIF-2 in crude liver preparations as assessed by Western blot analysis (data not shown). This indicated that the β-subunit in the purified protein had not been proteolytically degraded. The Mr = 17,000 protein observed in some preparations of two-subunit eIF-2 did not react with the antibody, which argues against, but does not exclude, the possibility that this protein was derived from the β-subunit.

The biological activity of rat liver eIF-2 was assessed by its ability to restore protein synthesis in hemin-deprived reticulocyte lysate (Fig. 3). The incorporation of [3H]leucine into protein was linear for at least 60 min in the presence of hemin, but was rapidly inhibited in its absence. The addition of increasing amounts of rat liver eIF-2 reversed this inhibition, with 0.8-1.5 pmol of globin synthesized for each pmol of eIF-2 added. Similar results were observed when the eIF-2 was added at zero time instead of at 16 min (data not shown). Results comparable to these were observed with reticulocyte eIF-2 (2).

**Association of Guanine Nucleotides with Rat Liver eIF-2**—The dissociation constant for GDP of rat liver eIF-2 was measured by Scatchard analysis (Fig. 4). The Kd of rat liver eIF-2 for GDP was 1.6 ± 1.0 × 10⁻⁷ M (n = 8) in the absence of Mg²⁺ compared to 2.2 ± 0.9 × 10⁻⁸ M (n = 5) in the presence of Mg²⁺. Under identical conditions, reticulocyte eIF-2 gave Kd values of 1.2 ± 0.3 × 10⁻⁷ M (n = 4) and 5.2 ± 0.6 × 10⁻⁸ M (n = 3), respectively. In both cases, the amount of GDP bound in the presence of Mg²⁺ was substantially reduced when compared to the amount bound when Mg²⁺ was not included in the assay. The decreased binding of [3H]GDP to eIF-2 in the presence of Mg²⁺ is probably due to GDP already bound to the purified factor. Siekierka and co-workers (21) demonstrated that homogeneous preparations of eIF-2 contain as

**FIG. 1.** SDS-polyacrylamide gel electrophoresis of eIF-2 from rat liver and rabbit reticulocytes. Samples were electrophoresed for 5 h at 30 V in a gel containing 12.5% acrylamide and 0.08% bisacrylamide and visualized by staining with Coomassie R-250 dye. Lane 1, rabbit reticulocyte eIF-2 (10 µg); lanes 2 and 3, three-subunit rat liver eIF-2 Step 4b (15 µg and 10 µg, respectively); lane 4, two-subunit rat liver eIF-2 (12 µg). The letters a, b, c, and d refer to the α-, β-, and γ-subunits of eIF-2 and a Mr = 17,000 protein, respectively.

**FIG. 2.** Immunoanalysis of rat liver and rabbit reticulocyte eIF-2. Left, rat liver and rabbit reticulocyte eIF-2 (5 µl) were spotted onto a nitrocellulose sheet in amounts ranging from 1–250 ng. Immunoanalysis was performed by sequential incubation of the nitrocellulose in 100 ml of: 1) TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl); 2) TBS containing 3% gelatin for 1 h; 3) rabbit anti-rat liver eIF-2 antibody diluted 1:1000 in TBS containing 1% gelatin for 25 h; and 4) 4 µCi of [3H]labeled Protein A in TBS containing 1% gelatin for 1 h. The nitrocellulose was washed with TBS three times between each step, and, following the last incubation, was washed two times additionally with water. [3H]-Labeled Protein A was visualized by autoradiography, spots were excised from the nitrocellulose sheet, and radioactivity was determined in a scintillation counter. Right, three-subunit rat liver eIF-2. A, rabbit reticulocyte eIF-2. Right, samples were electrophoresed in a gel containing 12.5% acrylamide and 0.08% bisacrylamide and then transferred from the gel to a nitrocellulose sheet in a Bio-Rad Transblot apparatus (60 V for 3 h). The nitrocellulose was processed as described above, with the exception that alkaline phosphatase-conjugated goat anti-rabbit IgG was substituted for [3H]-labeled Protein A in Step 4. The location of the eIF-2 subunits was visualized by soaking the blot in color development reagent containing 150 µg/ml 5-bromo-4-chloro-3-indolyl phosphate and 37.5 µg/ml nitro blue tetrazolium for 20 min at room temperature. The position of the subunits of eIF-2 is denoted in panel B as: a, a-subunit; b, β-subunit; c, γ-subunit; and d, Mr = 17,000 protein in the two-subunit eIF-2 preparation. Lanes 1, 4, 7, and 10, rabbit reticulocyte eIF-2 (0.3, 0.2, 0.1, and 0.05 µg, respectively); lanes 2, 5, 6, and 11; three-subunit rat liver eIF-2 Step 4b (0.42, 0.28, 0.14, and 0.07 µg, respectively); lanes 3, 6, 9, and 12; two-subunit rat liver eIF-2 (0.3, 0.2, 0.1, and 0.05 µg, respectively).
much as 9.5 pmol of GDP/mol of eIF-2. In the presence of Mg$^{2+}$, this unlabeled GDP would not exchange for exogenous [3H]GDP and so would account for the decreased amount of [3H]GDP bound under these conditions. In the presence of Mg$^{2+}$, rat liver two-subunit eIF-2 exhibited a $K_D$ for GDP that was identical with the three-subunit factor (2.3 × 10$^{-8}$ M; $n = 2$). The difference in $K_D$ between three-subunit liver and reticulocyte eIF-2 was statistically significant at $p < 0.05$. When GEF was included in the assay, the $K_D$ was increased 2.3-fold for two-subunit eIF-2 and 3.4-fold for the three-subunit factor.

Since the filter binding assay allowed only a short period of time to reach binding equilibrium, it was possible that the $K_D$ values obtained using this method were not true dissociation constants. To establish the validity of the filter binding assay, the dissociation constant of three-subunit liver eIF-2 was determined by equilibrium dialysis for 24 h over the same range of GDP concentrations. The $K_D$ obtained by equilibrium dialysis in the absence of Mg$^{2+}$ (1.4 × 10$^{-7}$ M; $n = 2$) was practically identical with that determined by the filter binding assay indicating that the latter assay reflected the actual $K_D$ of eIF-2 for GDP.

The dissociation constant of liver eIF-2 for GTP could not be measured by the filter binding assay. eIF-2 apparently formed an unstable complex with GTP, since no [3H]GTP above background could be detected on the nitrocellulose filters. Substitution of the GTP analogue, [3H]GMPPNP, for [3H]GTP in the assay also yielded an unstable binary complex with the initiation factor. These results were not due to an inability of eIF-2 to bind GTP or GMPPNP since eIF-2 could bind to either nucleotide in the presence of Met-tRNA.

Purification and Characterization of GEF from Rat Liver—

A summary of the purification of GEF from rat liver is shown in Table III. In the first 3 steps of the procedure, GEF was isolated as a GEF-eIF-2 complex, and its location in column fractions was identified by the ternary complex assay and confirmed by the GDP inhibition assay (Table IV). The GEF-eIF-2 complex eluted from the DEAE-cellulose column at a KCl concentration of 180 mM. Ternary complex activity ($440,000$) was inhibited greater than 50% by GDP preincubation (Table IV, Step 3a), indicating that GEF was also present in this fraction. In contrast, eIF-2 activity present in the $M_r = 440,000$ peak was inhibited greater than 50% by preincubation with GDP (Table IV, Step 3). This material either did not contain GEF or the eIF-2 present was not interacting with GEF to relieve the inhibition by GDP. In the
GEF purification from rat liver

The ribosomal supernatant from an eIF-2 preparation served as starting material for GEF purification. The activity is expressed as pmol of [35S]Met-tRNAi bound, which is actually a measure of eIF-2 activity. In early purification steps, this represents both free eIF-2 and GEF-eIF-2 complex. Since Sephacryl S-300 chromatography reflects primarily the GEF-eIF-2 complex. The low apparent yield following Sephacryl S-300 chromatography is attributed to the separation of GEF-bound eIF-2 from the GEF-eIF-2 complex.

2. DEAE-cellulose 0.037 0.037 1.00%
3. Sephacryl S-300 29.0 665.7
2. DEAE-cellulose 0.037 0.037 1.00%
3. Sephacryl S-300 29.0 665.7

GEF purification from rat liver

<table>
<thead>
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<th>Step</th>
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<td>1. Phosphocellulose</td>
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<td>3. Sephacryl S-300</td>
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<td>4. FPLC Mono Q</td>
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<tr>
<td>6. FPLC Superose 6</td>
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</table>

GEF inhibition of ternary complex assay

GEF samples (appropriately diluted) were incubated with an equal volume of 0.8 μM GDP or H2O at 28 °C for 4 min prior to the start of the ternary complex assay. Gel filtration chromatography on Sephacryl S-300 yielded two peaks of activity with apparent molecular weights of 1,250,000 (3a) and 440,000 (3b).

GEF activity was measured by the GDP exchange assay described under “Experimental Procedures” (open symbols); the absorbance at 280 nm was monitored as the sample eluted from the column (no symbols). The following proteins were chromatographed on the Superose 6 column as standards: ferritin, aldolase, bovine serum albumin, ovalbumin, cytochrome c. GEF activity eluted from the column before the highest molecular weight standard. Therefore, an accurate determination of the molecular weight could not be made. However, assuming that the standard curve could be extrapolated above the highest molecular weight standard, the apparent molecular weight of liver GEF was 750,000. GEF (42 μg) and eIF-2 (1 μg, Step 4b) were electrophoresed as described in Fig. 1 (right panel). The arrowheads on the right mark the expected positions of GEF subunits from reticulocytes based on reported molecular weights of 26, 39, 58, 67, and 82 x 10^3 (2).

DISCUSSION

eIF-2 has been isolated from rabbit reticulocytes and rat liver in both two- and three-subunit forms. The three-subunit form contains three dissimilar subunits (α, β, and γ), whereas

![Fig. 5. Superose 6 chromatography of GEF from rat liver.](image)

Following chromatography on Mono S, liver GEF was applied to a Superose 6 column at 0.4 ml/min. The column was developed with Buffer C containing 0.21 M KCl. Fraction collection was started after 7 ml of buffer had been applied to the column (left panel). GEF activity was measured by the GDP exchange assay described under “Experimental Procedures” (open symbols); the absorbance at 280 nm was monitored as the sample eluted from the column (no symbols). The following proteins were chromatographed on the Superose 6 column as standards: ferritin, aldolase, bovine serum albumin, ovalbumin, cytochrome c. GEF activity eluted from the column before the highest molecular weight standard. Therefore, an accurate determination of the molecular weight could not be made. However, assuming that the standard curve could be extrapolated above the highest molecular weight standard, the apparent molecular weight of liver GEF was 750,000. GEF (42 μg) and eIF-2 (1 μg, Step 4b) were electrophoresed as described in Fig. 1 (right panel). The arrowheads on the right mark the expected positions of GEF subunits from reticulocytes based on reported molecular weights of 26, 39, 58, 67, and 82 x 10^3 (2).
the two-subunit form invariably contains just the α- and γ-subunits. The identification of eIF-2 as a two-subunit protein can be explained in two ways. One explanation is that the mobility of the β-subunit during polyacrylamide gel electrophoresis varies considerably depending on the electrophoretic conditions utilized. Under certain conditions, the β- and γ-subunits co-migrate (5, 22). This problem can be avoided by using the modified Laemmli procedure described by Schreier et al. (22). The second explanation is based upon the susceptibility of the β-subunit to proteolysis during purification of eIF-2. Meyer et al. (23) have shown that, in rabbit reticulocytes, the β-subunit is particularly susceptible to proteolysis. If the proper precautions are not taken during eIF-2 preparation, the resulting protein can be almost completely deficient in β-subunit. In the purification of eIF-2 from rat liver, we have utilized a mixture of different proteases inhibitors chosen to prevent the action of a variety of protease classes including serine, thiol, and carboxyl proteases. The use of this mixture of inhibitors has allowed us to isolate eIF-2 from rat liver containing the three subunits characteristic of the factor isolated from other sources. The small amount of two-subunit material in the preparation can most likely be attributed to residual protease activity. This explanation is supported by two attempts to purify eIF-2 from commercially obtained rat liver rather than fresh liver. In both cases, only two-subunit eIF-2 was observed (data not shown). Similar findings have been reported for commercially obtained reticulocyte lysate (23). The report describing eIF-2 from liver as a two-subunit protein (9) is probably due to a loss of the β-subunit by proteolytic degradation during the purification procedure.

Structural similarity in the α-subunit of eIF-2 from the two sources is supported by Western blot analysis using antibodies raised against the liver protein. The antibodies demonstrate similar affinity for both reticulocyte and liver eIF-2α. In contrast, they show no recognition of the β-subunit of reticulocyte eIF-2. This, in conjunction with the difference in molecular weight, indicates that there are differences in the primary structure of the β-subunit from the two sources. Brown-Leudi and co-workers (13) have reported similar findings when comparing eIF-2 from HeLa cells with that from rabbit reticulocytes. The antibodies that they prepared against HeLa cell eIF-2 recognize both the α- and β-subunits of the HeLa cell factor. However, the recognition of the β-subunit of eIF-2 from reticulocytes is much weaker than that observed for the HeLa cell factor, whereas the reaction with the α-subunit is about the same.

The difference in KD of eIF-2 for GDP in the presence and absence of Mg2+ is not unexpected, based on the mechanism of GEF action. In the absence of Mg2+, GEF is superfluous since GDP bound to eIF-2 readily exchanges with free GDP (23). However, Mg2+ stabilizes the eIF-2-GDP binary complex, and GEF is required for the exchange of eIF-2-bound GDP for free GDP in the presence of Mg2+. This is reflected in the increase in KD of three-subunit eIF-2 for GDP in the presence of Mg2+ from 2.2 × 10^4 to 7.6 × 10^4 M when GEF is added to the assay. Since under these conditions GEF is simply reversing the Mg2+ stabilization of the eIF-2-GDP binary complex, it is reasonable that the KD for GDP is increased when Mg2+ is excluded from the assay. Analogous to these observations, the elongation factor EF-Tu has at least a 10-fold lower affinity for GDP in the absence of Mg2+ compared to the affinity in the presence of Mg2+ (24).

The difference in KD between liver and reticulocyte eIF-2 is unexpected since the physical and immunological properties of the α-subunits from the two sources are similar and the α-subunit is reported to bind guanine nucleotides (5). It is tempting to speculate that the differences in KD might be due to differences in the β-subunit since it demonstrates the greatest distinction between the two factors. A report showing that azido-GTP labels the β- as well as the α-subunit of eIF-2 (25) indicates that the β-subunit might be involved in guanine nucleotide binding. Although the similarity of KD of two- and three-subunit eIF-2 does not directly support this suggestion, neither does it exclude it, since the two-subunit factor does contain a peptide which could be a fragment of the β-subunit.

Two-subunit eIF-2 exchanges GDP much more slowly in the presence of GEF and Mg2+ than does three-subunit eIF-2. This difference is reflected in a decrease in the KD for GDP of two- versus three-subunit eIF-2. Together, these observations imply that the β-subunit of eIF-2 is required for maximal interaction of the initiation factor with GEF. Manchester has proposed that the exchange of GDP in the eIF-2-GDP binary complex for GTP is too slow in the absence of GEF to support active protein synthesis (26). The data presented here suggest that the function of the β-subunit of eIF-2, previously unknown, is to allow optimal GEF interaction with eIF-2 to catalyze guanine nucleotide exchange and therefore maintain the protein synthetic rate.

In summary, whereas the physical properties of the eIF-2 holoprotein from rat liver and rabbit reticulocytes are similar, there are significant differences between the two proteins. The primary structure of the β-subunit is likely to be different since both its molecular weight and antibody reactivity are different. The KD for GDP in the presence of Mg2+ is significantly different between the two proteins. Finally, whereas the direct involvement of the β-subunit in guanine nucleotide binding is still speculative, lack of this subunit does interfere with GEF interaction with eIF-2, and the ability of GEF to exchange GDP bound to eIF-2 for free GDP is impaired. Based on its behavior during column chromatography, GEF from rat liver must share some physical characteristics such as overall charge with the protein from rabbit reticulocytes. Likewise, based on the two GEF assays described here, it can be concluded that the factors from the two sources function in a similar manner in catalyzing the exchange of guanine nucleotides bound to eIF-2. However, the subunit composition and molecular weight of GEF from rat liver may differ considerably from the reticulocyte protein. Studies in progress should help in defining the roles of eIF-2 and GEF in the regulation of protein synthesis in rat liver.

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REFERENCES

Supplementary material to

Purification and Characterization of Eukaryotic Initiation Factor 4
and a Cytosine Nucleoside Exchange Factor from Rat (48)

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EXPERIMENTAL PROCEDURES

Materials, radioactive compounds (3H)-guanine, (3H)-UMP, and (3H)-GTP were purchased from American Corporation, rabbit liver RNA from Grand Island Biological Co., NHEPES, ATP, TRIS, and AMP-Antigen (Type II) from Pharmacia Fine Chemicals, Inc., phosphocellulose and DEAE-cellulose (type DE-52) from Whatman, Ltd., RNAse, purified puromycin, puromycin kinase, ATP, magnesium chloride, creatine phosphate, creatine phosphate kinase (EC 2.7.3.2), GTP and ATP (sonicated on dry ice) from Sigma Chemical Co., and Filtron X from National Diagnostics. Dichloro-dicyano-methane phos-
phate was a gift from Shell Development Corp.

Preparation of [3H]-Met-tRNA
Radioactive Met-tRNA was prepared by modification of the method described by Merriick et al. (28). Rabbit liver RNA was enzymatically phosphorylated with 13P-phosphate by incubation with bovine-heart-elongation-tRNA synthetase, which was prepared as described by Muehrer and Berg (29) with revision of the hydroxyproline volume. An affinity chro-
matography step on AMP-agarose (28) was added to reduce a desalination activity contaminating the synthetase preparation. Pilot assays were used to determine the optimal ratio of tRNA to synthetase in the charging assay. Each pilot assay contained 100 μl up to 175 μl of rabbit liver tRNA, up to 20 μg of synthosine, 100 μg [F6H]Met, 7.5 μg 10 mM MgCl2, and ATP, 1 mM DTT, 0.1 mM EDTA, and 0.5 mM MgCl2. After preincubation for 5 minutes, an ATP generating system consisting of 20 μM creatine phosphate, 10 units of creatine phosphokinase, and 10 units of pyruvate kinase. Addition of the ATP generating system to the assay decreased the yield of charged tRNA, up to 100%, presumably by preventing inhibition of the reaction by the accumulation of AMP. The reaction components were incubated for 40 min at 37° and the aminoacyl-
tRNA was assayed as described for TTP-preactivated radioactivity. After optimal con-
ditions were established, the pilot assays were scaled up to reaction mixtures of larger volume. These reaction mixtures were incubated at 37° for 60 min followed by the addition of 1/10 volume of 3N acetic acid, 0.1 N. This mixture was extracted with an equal volume of water-saturated phenol and applied at a flow rate of 75 μl x 2 x 75 cm of a 0/25 column equilibrated
with 0.1 M potassium phosphate. Fractions corresponding to the radioactive aminoacyl-tRNA were pooled and lyophilized. The resulting powder was dissolved in 10 μl of sodium acetate, pH 5.0, 1 mM dithiothreitol such that the concentration of [3H]-Met-tRNA was approximately 20 pmol/μl.

Before use, the charged tRNA was further purified by reverse phase chromatography on C18 columns (20). The charged tRNA was dialyzed 3 x 10 μl of 4 M NaCl, 10 μl magnesium acetate, 10 μl sodium acetate, pH 4.5, and applied at a flow rate of 30 ml/h to a 0.9 x 15 cm of IRDE-cellular column equilibrated with the same buffer. After washing the column with 0.4 M NaCl buffer, [3H]-Met-tRNA was eluted successively with 0.5 M NaCl, 10 mM magnesium acetate, 10 μl sodium acetate, pH 4.5, at a flow rate of 15 μl/h. Fractions containing charged tRNA, were pooled, deacidified, dialyzed against 0.1 M potassium phosphate, 0.1% bovine serum albumin, and analyzed for precipitation (20%) at -20°, and the final product suspended in 10 mM sodium acetate, pH 5.0, 1 mM dithiothreitol and stored in aliquots at -70°.

Assay of [3H]-tRNA Activity. Formation of a ternary complex of eIF-2, GTP, and Met-tRNA was measured by a modification of the method described by Smith and Chahwan (21). A 0.5 μl reaction mixture contained 10 mM MgCl2, pH 7.4, 100 μl 10 μM [3H]-Met-tRNA, 1 mM dithiothreitol, 0.2 mM GTP, 3.2 mPM potassium phosphate, 100 μg/ml bovine serum albumin, and 0.01 μM eIF-2 (Type II, Sigma Chemical Co.), and various amounts of eIF-2 activity. The mixture was incubated for 15 min at 30°, diluted with 2.5 ml of ice-cold wash buffer (50 mM MgCl2, pH 7.4, 2 mM magnesium acetate, 10 μl sodium acetate, pH 6.0), and assayed as described above. The [3H]-tRNA was then washed twice with ice-cold wash buffer and dissolved in 0.5 ml of EtOH x acetic acid wash buffer and stored in aliquots at -70°.

Assay of GEF Activity. GEF activity was assayed by one of two methods. The first method involved provision of GDP inhibition of ternary complex forma-
tion in the presence of 22% as described by Fiers and Denayer (3). Directly, samples (10 μl) were incubated at 30° for 6 min with an equal volume of 0.1 μM GDP prior to assay for ternary complex activity as described above. The other assay for GEF measured the exchange of the exchange of the GDP bound to eIF-2 for unlabelled GDP or GEF (6). Samples (10 μl) containing eIF-2 (0.1 μg, step 60) with or without GDP was preincubated with an equal volume of [35S]-GDP (0.1 μM, 10.8 Ci/mmol) at 0° for 5 minutes. The addition of 10 μl of 6 mM magnesium acetate followed immediately by 100 μl of buffer containing 50 mM MgCl2, pH 7.4, 1 mM ATP, 2 mM magnesium acetate, 1 mM dithio-
threitol, 0.2 mM potassium phosphate and albumin, and either 0.2 μM GDP or 0.2 μM GDP. The reaction was then incubated at 30° for various times, diluted with 2.5 ml of ice-cold wash buffer and filtered through a Millipore HAWP filter. The filters were processed as above for the eIF-2 assay.

Preparation of Liver Ribosomal Supernatant and Salt Wash Fractions. Male Sprague-Dawley rats were killed by cervical dislocation; livers were quickly removed and placed in ice-cold homogenization buffer (20 mM sucrose, 35 mM KCl, 1 mM MgCl2, 0.5 mM EDTA, 10 mM Tris-HCl, pH 7.4, 10 mM phosphoenolpyruvate, fluoride, 1 mg dichloro-
dicyano-methane phosphate, 0.4 μg ml insulin, 2 μg/ml chymo-
trypsin, 30 μg/ml leupeptin, and 30 μg/ml aprotinin). Livers were homogenized in 2/1 volume of homogenization buffer using a Polytron homogenizer (3 x 10 sec) set at 25% of maximum speed. The homogenate was centrifuged at 20,000 x g for 30 min at 4°. The resulting supernatant was centrifuged for 2 hr at 150,000 x g at 4°. The postribosomal supernatant was used for GEF purification as described below. The pellets were suspended in ice-cold homogenization buffer using a Polytron homogenizer (10 sec) set at 25% of maximum speed. The KCl con-
ncentration was adjusted to 0.5 M by the addition of 3 M KCl and the sus-
ant was allowed to stand for 60 min at 4°. The supernatant was then re-
centrifuged at 150,000 x g for 2 hr and the supernatant was dialyzed for 12 hr at 4° against buffer A (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.5 mM EDTA, and 10% glycerol) containing 100 mM KCl and 0.5 mM phenylmethylsul-
fonyl fluoride. The dialyzed sample was fractionated with ammonium sulfate and the material precipitating between 40 and 50% saturation was collected, dialyzed in buffer B in 0.25 M KCl (buffering 200 KCl) and dialyzed against a 60-fold excess of the same buffer. The ribosomal salt wash fraction was either used immediately for GEF purification or stored at -70°.

GEF Purification. All purification steps were carried out at 4°. The riboso-
mal salt wash fraction from 150 ml of rat liver was applied to a phenylhydrophi-
line column (2.6 x 15 cm) equilibrated with Buffer C (buffer with 20 mM MOPS, pH 7.5, instead of 40 mM Tris-HCl, pH 7.5) containing 250 mM KCl. The column was thoroughly washed with the same buffer and eIF-2 was eluted at 30 ml/h with a 50 ml linear KCl gradient (0.5 to 1.5 M KCl) in Buffer C. Fractions (10 ml) were collected and assayed for ternary complex activity. Those fractions with the greatest activity were pooled and concentrated during dialysis against Buffer C containing 50 mM KCl in a Micro-Prep II column (Bio-Molecular Dynamics). The dialyzed material was applied to a 5 x 12 cm column containing DEAE-cellulose that had been equilibrated in Buffer C containing 50 mM KCl in a Micro-Prep II column (Bio-Molecular Dynamics). After washing the column with the same buffer, the column was developed with a 200 ml linear gradient (50 to 400 ml 40 mM NaCl in Buffer C). Fractions (4 ml) that contained the greatest eIF-2 activity were pooled and concentrated to a final volume of 3 ml by dialysis against Buffer C containing 0.7 M KCl in a Micro-Precip II column. The concentrated sample was loaded on a 10% (w/v) sucrose cushion gradient (12 ml containing 0.7 M KCl, 0.1% (w/v) Brij-35, and 20 mM potassium phosphate, pH 7.5). The tubes were centrifuged in a SW-50 rotor at 300,000 rpm for 51 hr. Fractions (4 ml) that contained the greatest eIF-2 activity were pooled and the peak fractions of eIF-2 activity were pooled and concentrated during dialysis against Buffer C containing 50 mM KCl in a Micro-Prep II column. eIF-2 was stored in small aliquots at -70° until use.

Alternatively, eIF-2 was purified from rat liver using Fast Protein Liquid Chromatography (FPLC). The ribosomal salt wash fraction was fractionated with ammonium sulfate and subjected to ion exchange chromatography on phospho-
cellulose as described above. Following concentration and dialysis the sample was applied to a Mono Q HR 5/5 column equilibrated with Buffer C containing 10 mM KCl. The column was washed with Buffer C containing 180 mM KCl and then developed with a linear KCl gradient (180 mM to 400 mM KCl) in Buffer C. The fractions containing eIF-2 activity were pooled, diluted with 2 volumes of cold water and applied to a Mono S HR 5/5 column equilibrated with Buffer C containing 60 mM KCl. After washing the column with 180 mM KCl in Buffer C, eIF-2 was eluted from the column with a linear KCl gradient (180 mM to 400 mM KCl) in Buffer C. eIF-2 was concentrated, dialyzed and stored as described above.

GEF Purification. All purification steps were carried out at 4°C. Phosphocellulose was equilibrated with Buffer C containing 150 mM KCl, and 150 ml mixed volume of the ion exchange material was collected and partially dried by vacuum filtration on a Buchner funnel. The phosphocellulose was added to the post-nuclear supernatant from 150 g of rat liver and the mixture was slowly stirred at 4°C for 1 h. The phosphocellulose was collected by centrifugation (20,000 x g for 1 h) and washed 4 times with Buffer C containing 250 mM KCl by resuspending the ion exchange material in 300 ml of the buffer, stirring for 15 min, and collecting by centrifugation. GEF and eIF-2 were eluted with Buffer C containing 0.5 M KCl. The 0.5 M KCl wash was concentrated by diafiltration overnight against saturated ammonium sulfate containing 50 mM 2-mercaptoethanol. The dialyzed material was centrifuged, resuspended in Buffer B containing 50 mM KCl, and dialyzed against the same buffer overnight at 4°C. GEF-eIF-2 was eluted onto a DEAE-cellulose column (2.5 x 30 cm) at a flow rate of 60 ml/h. The column was washed with 200 ml of Buffer C with 50 mM KCl and a 500 ml linear gradient from 50 mM to 400 mM KCl in Buffer C was applied to the column at a flow rate of 30 ml/h. The GEF-eIF-2 complex was located in the column fractions (10 ml) by its ability to form a ternary complex with GDP and [35S]Met-tRNA. The presence of GEF was confirmed using the GDP inhibition assay described above. Three fractions with ternary complex activity were pooled and concentrated during dialysis against Buffer B with 50 mM KCl in a Micro-ProDCel. The dialyzed sample was loaded onto a 2.6 x 70 cm Sephacryl 16/500 column and eluted with Buffer C containing 250 mM KCl at a flow rate of 15 ml/h. 4 ml fractions were collected. Two peaks of eIF-2 activity were observed; only one contained GEF activity. Fractions containing the two peaks were pooled separately and concentrated during dialysis in the Micro-ProDCel. GEF-eIF-2 was applied to a Mono Q HR 5/5 column equilibrated with 10 mM KCl in Buffer C. The column was washed with Buffer C containing 150 mM KCl and then developed with a linear KCl gradient (110 mM to 400 mM KCl) in Buffer C containing 50 mM KCl. The presence of GEF in the column fractions was measured by the GDP-exchange assay as described above. The fractions containing GEF activity were pooled, dialyzed with 3 volumes of cold water and applied to a Mono S HR 5/5 column equilibrated with Buffer C containing 60 mM KCl. After washing the column with 80 mM KCl in Buffer C, GEF was eluted from the column with a linear KCl gradient (30 mM to 400 mM KCl) in Buffer C. Fractions containing GEF activity were pooled and concentrated in the Micro-ProDCel and then gel-filtered on a Sephacryl S-300 column. Following dialysis and concentration in the Micro-ProDCel, GEF was stored in small aliquots at -70°C until use.