Purification and Characterization of Bacterially Expressed Mammalian Translation Initiation Factor 5 (eIF-5): Demonstration That eIF-5 Forms a Specific Complex with eIF-2†

Jayanta Chaudhuri,‡ Kallol Das,‡ and Umadas Maitra*

Department of Developmental and Molecular Biology, Division of Biology, Albert Einstein College of Medicine of Yeshiva University, Jack and Pearl Resnick Campus, Bronx, New York 10461

Received October 21, 1993; Revised Manuscript Received February 3, 1994®

ABSTRACT: Eukaryotic translation initiation factor 5, eIF-5, has been purified to apparent electrophoretic homogeneity from overproducing *Escherichia coli* cells expressing the cDNA of the initiation factor under the control of the T7 promoter–T7 RNA polymerase system. Purified recombinant eIF-5 mimics natural eIF-5 isolated from mammalian cells in size, in specific activity, in its ability to catalyze the hydrolysis of GTP bound to the 40S initiation complex, and in the subsequent joining with 60S ribosomal subunits to form the 80S initiation complex. Further characterization of eIF-5 demonstrates that eIF-5 specifically associates with eIF-2, forming an eIF-2·eIF-5 complex. The protein complex sediments in glycerol gradients with an apparent M_r of 160 000, suggesting that the two proteins associate in a 1:1 stoichiometry. The association between the two initiation factors is highly specific. Addition of ^{32}P-labeled eIF-5 to a partially purified rabbit reticulocyte initiation factor preparation that contained, in addition to eIF-2 and eIF-5, other initiation factors and many other proteins resulted in the specific binding of labeled eIF-5 only to eIF-2, forming a 160-kDa protein complex. In agreement with these observations, we found that in crude initiation factor preparations derived from rabbit reticulocyte lysates, eIF-5 was present as an eIF-2·eIF-5 complex. The significance of eIF-2·eIF-5 complex formation in the overall mechanism of GTP hydrolysis in protein synthesis initiation is discussed.

Initiation of protein synthesis in eukaryotic cells occurs by a series of discrete partial reactions requiring the participation of a large number of specific protein factors, termed initiation factors. An obligatory intermediate step in the initiation of protein synthesis in eukaryotes is the GTP-dependent binding of initiator Met-tRNA$_f$ and mRNA to a 40S ribosomal subunit to form the 40S initiation complex (40S·mRNA·Met-tRNA$_f$·eIF-2·GTP). A 60S ribosomal subunit then joins the 40S initiation complex to form the 80S initiation complex (80S·mRNA·Met-tRNA$_f$) that is active in peptidyl transfer [for reviews, see Maitra *et al.* (1982), Kozak (1983, 1992), Hershey (1991), and Merrick (1992)]. The subunit-joining reaction specifically requires the participation of eukaryotic initiation factor 5 (eIF-5),[1] a monomeric protein that migrates on SDS gels as a 58-kDa protein (Raychaudhuri *et al.*, 1985a, 1987; Ghosh *et al.*, 1989). It has been demonstrated that eIF-5 interacts with the 40S initiation complex to promote the hydrolysis of ribosome-bound GTP. GTP hydrolysis is essential for the release of eIF-2 and guanine nucleotide (as an eIF-2·GDP complex) and the subsequent joining of the 60S ribosomal subunit to form a functional 80S initiation complex (Trachsel & Staehelin, 1978; Peterson *et al.*, 1979; Raychaudhuri *et al.*, 1985b; Chakrabarti & Maitra, 1991, 1992).

To date, only limited studies have been carried out on the structural and detailed functional aspects of eIF-5. This is in part because of the relatively low abundance of eIF-5 in eukaryotic cells and consequent inability to obtain sufficient quantities of the pure protein in stable form (Merrick, 1992; Raychaudhuri *et al.*, 1985a, 1987; Merrick *et al.*, 1975; Schreier *et al.*, 1977; Benne *et al.*, 1978). Recently, however, a 3.55 kilobase pair rat cDNA encoding functional eIF-5 has been cloned and sequenced (Das *et al.*, 1993). The entire coding region of the rat cDNA, with a calculated molecular mass of 48 926 daltons, was also expressed in *Escherichia coli* using the T7 promoter–T7 RNA polymerase system. Analysis of the expressed protein in crude cell-free extracts indicated the formation of a catalytically active eIF-5 protein that migrated on SDS gels with the same mobility as purified eIF-5 isolated from rabbit reticulocyte lysates (apparent M_r = 58 000) (Das *et al.*, 1993).

In this paper, we describe the purification of recombinant eIF-5 protein from overproducing bacterial cells, and its characterization. The results demonstrate that bacterially-expressed recombinant eIF-5 has properties that are similar to eIF-5 isolated from rabbit reticulocyte lysates. The ready availability of purified protein in relatively large quantities has also allowed us to carry out additional experiments that demonstrate that eIF-5 forms a specific complex with eIF-2.

EXPERIMENTAL PROCEDURES

tRNA, Purified Proteins, and Antibodies. The preparations of ^{35}S- or ^{3}H-labeled rabbit liver Met-tRNA$_f$ (10 000–30 000 cpm/pmol), ribosomal subunits from *Artemia salina* eggs, and purified eIF-2 and eIF-5 from rabbit reticulocyte lysates were as described in previous publications from this laboratory (Chakrabarti & Maitra, 1991, 1992; Chevesich *et al.*, 1993).

† This work was supported by Grant GM15399 from the National Institutes of Health and by Cancer Core Support Grant P30CA13330 from the National Cancer Institute.

* Correspondence should be addressed to this author.

‡ Both authors contributed equally to the work presented here.

® Abstract published in *Advance ACS Abstracts,* April 1, 1994.

[1] Abbreviations: eIF-2 and eIF-5, eukaryotic (translation) initiation factors 2 and 5, respectively; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl β-D-thiogalactopyranoside.

0006-2960/94/0433-4794$04.50/0

The specific activity of homogeneous eIF-2 was about 6000 units/mg of protein while that of homogeneous eIF-5 was 1.9 × 10^6 units/mg of protein. Definition of units for eIF-2 and eIF-5 was as described previously (Chakrabarti & Maitra, 1991; Chevesich *et al.*, 1993). Purified casein kinase II was isolated from the postribosomal supernatant of rabbit reticulocyte lysates by the procedure of Hathaway *et al.* (1979) as described (Ghosh *et al.*, 1989). The 40S initiation complex (40S·AUG·Met-tRNA$_f$·eIF-2·GTP), containing [^{35}S]Met-tRNA$_f$ (50 cpm/pmol) and [γ-^{32}P]GTP (10 000 cpm/pmol), was prepared and isolated free of unreacted reaction components by sucrose gradient centrifugation as described (Chakrabarti & Maitra, 1991, 1992). The preparation of specific rabbit antisera against purified denatured eIF-5 and affinity purification of anti-eIF-5 antibodies were described (Ghosh *et al.*, 1989).

Purification of Bacterially-Expressed eIF-5. The pET-5a-eIF-5 expression vector (designated pETIF5) (Das *et al.*, 1993) containing the eIF-5-coding sequence under the control of the strong T7 RNA polymerase promoter was used to transform *E. coli* strain BL21DE3 cells (Novagen) that carry the inducible T7 RNA polymerase gene in their chromosome under the control of *lacUV5* promoter (Studier *et al.*, 1990). A single colony isolated as an ampicillin-resistant transformant was grown to mid-logarithmic phase in 5 mL of 2YT medium (Sambrook *et al.*, 1989) containing 50 μg/mL ampicillin. The cells were then diluted 1:1000 into 2.5 L of 2YT medium containing 50 μg/mL ampicillin and grown to about 1.2 A_{600}, induced with 1 mM IPTG, and grown for an additional 3 h. The cells were centrifuged, washed with ice-cold 0.9% NaCl, quick-frozen in a dry ice–ethanol bath, and stored at −70 °C until use. The cell yield was about 12 g (wet weight). All purification procedures were carried out at 0–4 °C unless otherwise stated.

The frozen cells (12 g) were suspended in 36 mL of a buffer containing 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 30 mM KCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.5 mM phenylmethanesulfonyl fluoride and disrupted by sonication. After the cell debris was removed by centrifugation at 15000*g* for 10 min, the supernatant was treated with 30 μg of pancreatic DNase, incubated at 0 °C for 30 min, and then centrifuged at 48 000 rpm for 150 min in a Beckman 50 Ti rotor. The postribosomal supernatant (37 mL containing 148 mg of protein) was adjusted to 0.25 M KCl by adding 2 M KCl and then loaded onto a 28-mL bed volume of a DEAE-cellulose column equilibrated in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol (buffer A) + 0.25 M KCl. The unabsorbed proteins were washed from DEAE-cellulose with buffer A + 0.25 M KCl, and fractionated with solid ammonium sulfate. The proteins precipitating between 50 (291 g/L) and 70% saturation (additional 125 g/L) were dissolved in 4 mL of buffer A + 70 mM KCl containing 0.5 mM phenylmethanesulfonyl fluoride and dialyzed overnight against 1 L of the same buffer. The dialyzed protein fraction was clarified by centrifugation in a Sorvall centrifuge and applied to a column (6.5-mL bed volume) of DEAE-Sephacel equilibrated in buffer A + 90 mM KCl. The column was washed with this buffer until the A_{280} of the effluent was below 0.1. A linear gradient of 52-mL total volume from buffer A + 90 mM KCl to buffer A + 450 mM KCl was then applied to the column, and 1.5-mL fractions were collected. eIF-5 activity eluted at about 250 mM KCl concentration. Fractions containing eIF-5 were pooled (about 10 mL), adjusted to pH 6.8 by adding 0.5 M potassium phosphate, pH 6.0, and then loaded onto a 2.4-mL bed volume column of hydroxylapatite (Bio-Gel HTP, Bio-Rad) equilibrated with 20 mM potassium phosphate, pH 6.8, 1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol (buffer B). The column was then successively washed with 10-mL volumes each of 20, 100, 150, and 400 mM potassium phosphate, pH 6.8. (All buffer solutions used in the hydroxylapatite step also contained 1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol.) Approximately 1-mL fractions were collected. Fractions containing eIF-5 which eluted with 400 mM potassium phosphate were pooled, dialyzed for about 5 h against 500 mL of buffer B + 70 mM KCl to decrease the ionic strength of the protein fraction to that of buffer B + 100 mM KCl, and then applied to a 1-mL bed volume column of phosphocellulose that had been previously equilibrated with buffer B + 100 mM KCl. After the column was washed with 8 mL of the same buffer, a linear gradient of 20-mL total volume from buffer B + 100 mM KCl to buffer B + 800 mM KCl was applied. Fractions of 0.5 mL were collected in siliconized Eppendorf tubes and assayed for eIF-5 activity. Active fractions were pooled, dialyzed for about 7 h against 500 mL of 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 60% glycerol, and stored at −20 °C. Under these conditions, eIF-5 activity was stable for at least 6 months without significant loss of activity.

Isolation of a Partially Purified eIF-2/eIF-5 Preparation from Rabbit Reticulocyte Lysates. A partially purified eIF-2/eIF-5 preparation was isolated from rabbit reticulocyte lysates by subjecting the 0.5 M KCl wash proteins (120 mg) of the polysomal pellets through successive batchwise elutions from a DEAE-cellulose column and then a phosphocellulose column as described (Chevesich *et al.*, 1993). The dialyzed phosphocellulose eluate was stored in small aliquots at −70 °C. The specific activities of eIF-2 and eIF-5 in this preparation were about 500 and 2 × 10^5 units/mg of protein, respectively. On the basis of the specific activities of homogeneous eIF-2 and eIF-5 indicated before, these two initiation factors are only about 10% pure in these partially purified initiation factor preparations.

Preparation and Isolation of ^{32}P-Labeled eIF-5. Recombinant eIF-5 was phosphorylated with [γ-^{32}P]ATP (10 000–50 000 cpm/pmol) and casein kinase II in a reaction mixture similar to that described previously for phosphorylation of mammalian eIF-5 (Chakrabarti & Maitra, 1991). [^{32}P]eIF-5 was subsequently isolated by subjecting the reaction mixture to phosphocellulose chromatography as described (Chakrabarti & Maitra, 1991).

Assay of eIF-5 Activity. Two different assay methods, described in detail in a previous paper from this laboratory (Chevesich *et al.*, 1993), were employed to measure eIF-5 activity. In method A, the factor activity was measured by its ability to promote the joining of 60S ribosomal subunits to a preformed 40S initiation complex containing bound [^{35}S]-Met-tRNA$_f$ to form the 80S initiation complex. The formation of the 80S initiation complex containing bound [^{35}S]Met-tRNA$_f$ was determined by centrifugation in a 5–25% (w/v) sucrose gradient as described previously (Raychaudhuri *et al.*, 1985a, 1987). In method B, eIF-5 activity was measured by the ability of the factor to catalyze the hydrolysis of [^{32}P]-GTP bound to the 40S initiation complex (40S·AUG·Met-tRNA$_f$·eIF-2·[γ-^{32}P]GTP) (Chakrabarti & Maitra, 1991). The release of [^{32}P]P$_i$ by the cleavage of [γ-^{32}P]GTP bound to the 40S initiation complex was measured by the ammonium phosphomolybdate method of Conway and Lipmann (1964) as described by Dubnoff and Maitra (1972).

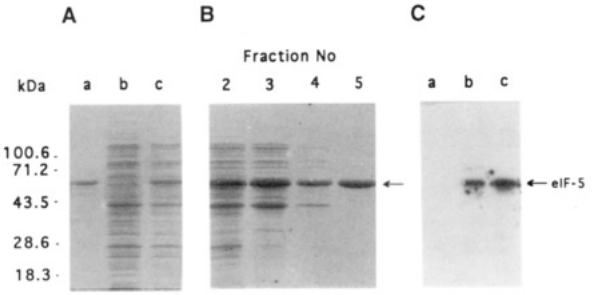

FIGURE 1: Purification of recombinant eIF-5 expressed in *E. coli*. *Panel A*: Expression of eIF-5 in *E. coli*. Proteins (5 μg each) from cell-free extracts of *E. coli* cells harboring the eIF-5 expression plasmid pETIF5 (lane c) as well as the parental vector pET-5a (lane b) were separated by 10% SDS–PAGE followed by Coomassie blue staining. A set of marker proteins of known molecular weights was run in a single parallel lane (not shown). In lane a, ~0.5 μg of purified rabbit reticulocyte eIF-5 was electrophoresed and then stained with Coomassie blue. *Panel B*: Samples of recombinant eIF-5 (5 μg each from fractions 2 and 3; 2 μg each from fractions 4 and 5 of Table 1) were resolved by SDS–PAGE (10% gel) and then visualized by Coomassie blue staining. All samples shown in panels A and B were run in the same gel. The arrow indicates the position of migration of purified eIF-5. *Panel C*: Approximately 0.2 μg of purified rabbit reticulocyte eIF-5 (lane b) or purified recombinant eIF-5 (lane c) and no eIF-5 (lane a) was phosphorylated in separate reaction mixtures with [γ-^{32}P]ATP (10 000 cpm/pmol) and casein kinase II (0.1 μg) as described under Experimental Procedures. The reactions were terminated by adding 10% TCA, and the precipitated proteins were analyzed by SDS–PAGE (10% gel) followed by autoradiography as described previously (Ghosh *et al.*, 1989).

Other Methods. eIF-2 activity was measured by the ability of the factor to promote GTP-dependent binding of [^{35}S]-Met-tRNA$_f$ to a nitrocellulose membrane filter as described previously (Stringer *et al.*, 1979). Protein was determined by the method of Bradford (1976) using reagents obtained from Bio-Rad Laboratories. SDS–polyacrylamide gel electrophoresis was carried out by an adaptation of the method described by Schreier *et al.* (1977). Immunoblot analysis of eIF-5 was performed using rabbit polyclonal anti-eIF-5 antibodies as probes as described by Ghosh *et al.* (1989).

RESULTS

Purification and Characterization of Recombinant eIF-5. Induction of *E. coli* BL21DE3/pETIF5 cells, harboring the pET5a-eIF-5 expression plasmid, with IPTG resulted in the synthesis of a 58-kDa polypeptide corresponding to the known size of eIF-5 isolated from mammalian cells [Figure 1, panel A, and Das *et al.* (1993)]. We observed that maximum level of eIF-5 was produced when the cells were induced at an A_{600} of 1.2. It can be calculated that approximately 8–10% of the total soluble proteins produced under these conditions was eIF-5 as judged by assay of eIF-5 activity as well as by densitometric tracing (not shown) of the Coomassie blue-stained SDS gel of Figure 1, panel A. Recombinant eIF-5 was purified to apparent electrophoretic homogeneity from these *E. coli* cells by a simple and rapid procedure as described under Experimental Procedures and summarized in Figure 1 and Table 1.

eIF-5 was monitored at different purification steps by SDS–PAGE followed by Coomassie blue staining (Figure 1, panel B) as well as by immunoblot analysis of eIF-5 fractions using rabbit polyclonal anti-eIF-5 antibodies (data not shown). Fractions exhibiting intense immunoreactive eIF-5 polypeptide were pooled and assayed for eIF-5 activity (Table 1). A yield of about 4 mg of pure protein was obtained from 12 g (wet weight) of *E. coli* cells. The size (Figure 1, panel B) and the specific activity of the purified enzyme (1.6×10^6 units/mg) (Table 1) were similar to those of homogeneous eIF-5 isolated from rabbit reticulocyte lysates (Chevesich *et al.*, 1993). Recombinant eIF-5 can be phosphorylated by casein kinase II (Figure 1, panel C, lane c). Phosphorylation of recombinant eIF-5 by casein kinase II did not affect its activity in catalyzing the joining of 60S ribosomal subunits to the preformed 40S initiation complex (data not shown).

Table 1: Purification of eIF-5 from *E. coli* BL21 Cells Carrying the Recombinant eIF-5 Expression Plasmid pETIF5

fraction	total protein (mg)	total units[a]	sp act. (units/mg of protein)
(1) crude postribosomal supernatant	148	2×10^7	1.3×10^5
(2) ammonium sulfate	30	1.2×10^7	4×10^5
(3) DEAE-Sephacel	9.6	7.6×10^7	8×10^5
(4) hydroxylapatite	4.0	6×10^6	1.5×10^6
(5) phosphocellulose	3.4	5.4×10^6	1.6×10^6

[a] One unit of eIF-5 is defined as the activity required to promote the formation of 1 pmol of 80S initiation complex by the joining of 60S ribosomal subunits to a preformed 40S initiation complex.

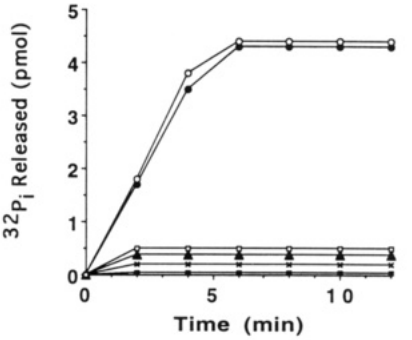

FIGURE 2: Hydrolysis of GTP bound to the 40S initiation complex. Reaction mixtures (70 μL each) contained 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, and isolated 40S initiation complex (40S·AUG·Met-tRNA$_f$·eIF-2·[γ-^{32}P]GTP) containing 4.2 pmol of bound [γ-^{32}P]GTP (10 000 cpm/pmol) and 2 ng of purified recombinant or mammalian eIF-5. Where indicated, free [γ-^{32}P]GTP (10 pmol) and purified eIF-2 (2 μg) replaced isolated 40S initiation complex as substrates for recombinant eIF-5-mediated GTP hydrolysis reactions. Incubation was at 20 °C. Aliquots (10 μL) were removed at various times as indicated, and the amount of [^{32}P]P_i released by the hydrolysis of [γ-^{32}P]GTP was measured by the ammonium phosphomolybdate method of Conway and Lipmann (1964) as described by Dubnoff and Maitra (1972). Reaction mixtures lacking eIF-5 were also incubated. The amount of [^{32}P]P_i released in these control reaction mixtures was <0.05 pmol and has not been subtracted from the results shown. The results shown in the figure represent the total amount of [^{32}P]P_i formed per 70-μL reaction mixture. The substrates used in various reactions were as follows: (○) 40S initiation complex + mammalian eIF-5; (●) 40S initiation complex + recombinant eIF-5; (□) 40S initiation complex only; (■) GTP only; (×) recombinant eIF-5 + GTP; (▲) recombinant eIF-5 + eIF-2 + GTP. It should also be noted that control reaction mixtures (not shown in the figure) containing (a) [γ-^{32}P]GTP, purified 40S subunits (0.1 A_{260} unit), and Met-tRNA$_f$ (8 pmol) and (b) [γ-^{32}P]-GTP, 40S subunits (0.1 A_{260} unit), and eIF-2 were also incubated in the presence or absence of eIF-5. The amount of P_i released in each of these control reaction mixtures was <0.1 pmol as described previously (Chakrabarti and Maitra, 1991).

Bacterially-expressed eIF-5 promoted rapid and quantitative hydrolysis of GTP bound to the 40S initiation complex (40S·AUG·Met-tRNA$_f$·eIF-2·[γ-^{32}P]GTP) (Figure 2), but only when the nucleotide was bound to the 40S initiation complex. eIF-5 did not hydrolyze either free GTP or GTP in the presence of eIF-2 (Figure 2). These properties of recombinant eIF-5 are thus similar to those reported (Chakrabarti & Maitra, 1991) for eIF-5 isolated from mammalian cells. When the initiation reaction mixtures contained both the 40S initiation complex and 60S ribosomal subunits, purified recombinant eIF-5, like natural eIF-5 (Raychaudhuri *et al.*,

1985a, 1987; Ghosh *et al.*, 1989), strongly stimulated the formation of the 80S initiation complex (data not shown).

Association of eIF-5 with eIF-2. We have recently observed (Chevesich *et al.*, 1993) that although homogeneous eIF-5 isolated from rabbit reticulocyte lysates is a monomeric protein of about 58 kDa, in partially purified eIF-5 preparations the 58-kDa eIF-5 remained bound to another cellular protein and sedimented in glycerol gradient centrifugations as a protein of about 160 kDa. Similar observations were also made with eIF-5 isolated from the yeast *Saccharomyces cerevisiae* (Chakravarti *et al.*, 1993). We undertook to identify and characterize the protein to which eIF-5 is bound in cruder rabbit reticulocyte initiation factor preparations. Since eIF-5 functions in the initiation of protein synthesis by its interaction with the Met-tRNA$_f$·eIF-2·GTP ternary complex bound to the 40S initiation complex (Chakrabarti & Maitra, 1991), the possibility existed that eIF-5 might interact with eIF-2. The observed molecular weight of 160 000 of the eIF-5 protein complex in cruder preparations, close to the sum of the molecular weights of eIF-2 (M_r = 120 000) and eIF-5 (M_r = 48 926), also suggested such an interaction.

To test this possibility, we prepared a partially purified eIF-2/eIF-5 preparation by chromatography of the 0.5 M KCl wash proteins of rabbit reticulocyte polysomes through a succession of DEAE and phosphocellulose columns as described under Experimental Procedures and then subjected the protein preparation to glycerol gradient centrifugation in a buffer containing 100 mM KCl (Figure 3). Assay of the gradient fractions for eIF-2 and eIF-5 activities showed that the two initiation factors cosedimented at a position corresponding to an M_r = 160 000 (Figure 3, panel A). In contrast, when purified eIF-2 and eIF-5 were separately subjected to glycerol gradient centrifugation under similar conditions, eIF-2 and eIF-5 sedimented at positions expected for purified eIF-2 (M_r = 120 000) and purified eIF-5 ($M_r \cong$ 50 000), respectively (Figure 3, panel B).

Further confirmation of the association between eIF-2 and eIF-5 was obtained by incubating purified rabbit reticulocyte eIF-2 with purified recombinant eIF-5 at 0 °C and then analyzing the reaction mixture by glycerol gradient centrifugation. Under these conditions, the two initiation factors cosedimented at a position corresponding to an M_r = 160 000 (Figure 4). The molecular weight value of this complex is close to the sum of the molecular weights of eIF-2 (M_r = 120 000) and eIF-5 (M_r = 50 000), indicating that complex formation between the two initiation factors occurs in a 1:1 stoichiometry.

To facilitate further studies on eIF-2·eIF-5 complex formation, we labeled eIF-5 with ^{32}P using [γ-^{32}P]ATP and casein kinase II (Figure 1, panel C). We also used Sephadex G-200 gel filtration to investigate eIF-2·eIF-5 complex formation. As shown in Figure 5, when ^{32}P-labeled eIF-5 was incubated with eIF-2 and the reaction mixture analyzed by Sephadex G-200 gel filtration, all the ^{32}P radioactivity eluted from the column in the excluded volume. In the absence of eIF-2, however, [^{32}P]eIF-5 eluted from the column at the position expected for a protein of about 50 kDa (Figure 5). The association between eIF-5 and eIF-2 is highly specific. Incubation of [^{32}P]eIF-5 with multisubunit initiation factors eIF-3 or eIF-2B did not result in the association of eIF-5 with either factor (Figure 5). 40S and 60S ribosomal subunits as well as several control proteins, e.g., bovine serum albumin, thyroglobulin, ovalbumin, myoglobin, and cytochrome *c*, were also inactive in complex formation with [^{32}P]eIF-5 (data not shown). Prior incubation of eIF-2 with either GTP or GTP

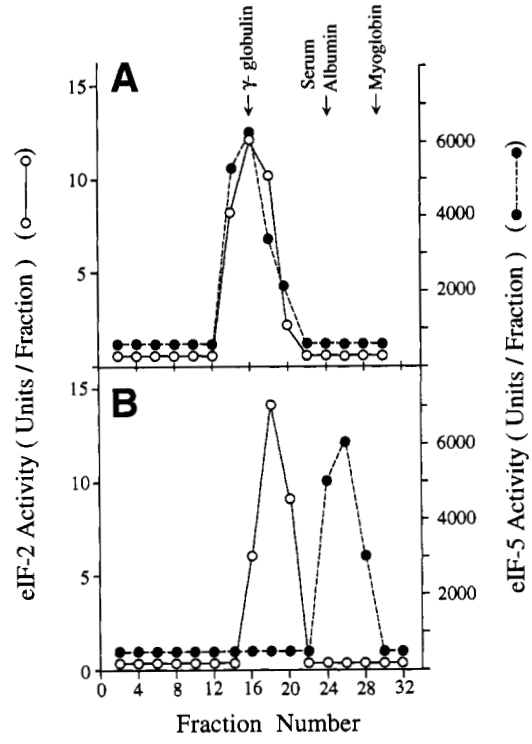

FIGURE 3: Analysis of a partially purified rabbit reticulocyte eIF-2/eIF-5 preparation by glycerol gradient centrifugation. *Panel A*: A partially purified eIF-2/eIF-5 preparation (200 μg containing 40 000 units of eIF-5 and 100 units of eIF-2 activities) prepared as described under Experimental Procedures was layered onto 11-mL linear 15–40% (v/v) glycerol gradients containing 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 0.2 mM phenylmethanesulfonyl fluoride, and 100 mM KCl. *Panel B*: Similar amounts of purified rabbit reticulocyte eIF-2 and eIF-5 were also separately layered onto two 11-mL linear 15–40% (v/v) glycerol gradients. A set of known molecular weight marker proteins was run in a fourth tube. All centrifugation analyses were carried out in the same SW41 rotor for 50 h at 40 000 rpm at 2 °C. Fractions of 0.35 mL were collected from the bottom of each tube and assayed for eIF-5 and eIF-2 activities as described under Experimental Procedures.

and Met-tRNA$_f$ had no effect on complex formation between eIF-2 and [^{32}P]eIF-5 (data not shown). Furthermore, incubation of eIF-2 with a nonspecific control protein such as bovine serum albumin did not result in complex formation between eIF-2 and albumin as judged by Sephadex G-200 gel filtration analysis (data not shown). The specificity of complex formation between eIF-5 and eIF-2 was further demonstrated by showing that when ^{32}P-labeled eIF-5 was incubated with a relatively crude initiation factor preparation containing eIF-2, ^{32}P radioactivity cosedimented with eIF-2 at a position corresponding to an M_r = 160 000 (Figure 6). Since the protein preparation used in this experiment contained, in addition to eIF-2 and eIF-5, other initiation factors and many other proteins, these results demonstrate that ^{32}P-labeled eIF-5 specifically associated with eIF-2 present in the protein preparation used. Furthermore, the sedimentation of both eIF-2 and eIF-5 as a sharp peak of activity at a position corresponding to about 160 kDa in glycerol gradient centrifugation of crude initiation factor preparations (Figures 3 and 6) suggests that the interaction between eIF-2 and eIF-5 is highly specific. Neither factor nonspecifically associates with other proteins present in these crude protein preparations.

DISCUSSION

Previous observations made in our laboratory (Chakrabarti & Maitra, 1991) showed that eIF-5 catalyzes the hydrolysis

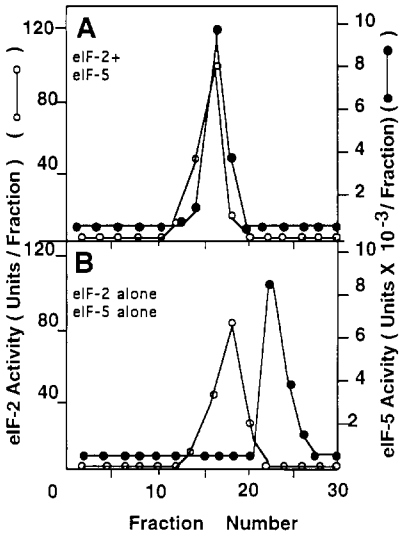

FIGURE 4: Analysis of eIF-2·eIF-5 complex formation. A reaction mixture (70 μL) (panel A) containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 5% glycerol, purified (6000 units/mg of protein) rabbit reticulocyte eIF-2 (75 μg), and purified recombinant eIF-5 (30 μg) was incubated at 0 °C for 1 h. The mixture was then layered onto a 11-mL 15–40% (v/v) glycerol gradient containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM EDTA, and 1 mM dithiothreitol and centrifuged in a Beckman SW41 rotor at 38 000 rpm for 55 h. Two other reaction mixtures (panel B), one containing 75 μg of eIF-2 alone and another containing 30 μg of eIF-5 alone, were incubated and analyzed in separate tubes in the same rotor. A set of molecular weight marker proteins was run in a fourth tube. It should be noted that prior to use the centrifuge tubes were presoaked in a buffer containing 1 mg/mL bovine serum albumin. Following centrifugation, fractions of 0.35 mL were collected from the bottom of each tube and assayed for eIF-2 and eIF-5 activities.

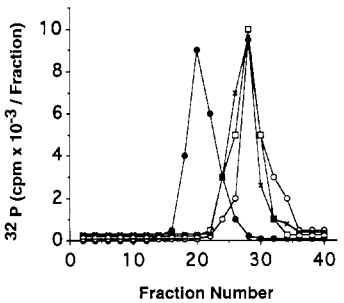

FIGURE 5: Analysis of the specificity of eIF-2·eIF-5 complex formation by Sephadex G-200 gel filtration. Reaction mixtures (175 μL) contained buffer A (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol), approximately 0.5 μg of ^{32}P-labeled recombinant eIF-5 containing 60 000 cpm of radioactivity, and, where indicated, 3 μg of either purified eIF-2 or other initiation factors, eIF-3 (27 μg) or eIF-2B (15 μg). (The molar ratio of eIF-5 to eIF-2 was about 1:2 while that of eIF-5 to eIF-3 or eIF-2B was about 1:5.) Following incubation for 1 h at 0 °C, the formation of a complex between eIF-5 and the other initiation factor added was analyzed by Sephadex G-200 gel filtration as follows. The reaction mixture was applied to a column (0.4 × 25 cm, 12-mL bed volume) of Sephadex G-200 previously equilibrated in buffer A containing 5% glycerol. The column was developed with the same equilibration buffer. Fractions (200 μL) were collected and counted in Aquasol (DuPont–New England Nuclear) in a liquid scintillation spectrometer. (●) [^{32}P]eIF-5+eIF-2; (○) [^{32}P]eIF-5 alone; (×) [^{32}P]-eIF-5+eIF-3; (□) [^{32}P]eIF-5+eIF-2B.

of GTP only when the nucleotide is bound to eIF-2 as a Met-tRNA$_f$eIF-2·GTP ternary complex on the 40S ribosomal subunit. The factor does not hydrolyze either free GTP or GTP bound in a Met-tRNA$_f$eIF-2·GTP ternary complex in the absence of 40S ribosomal subunits. These results suggest that the protein–protein interaction between eIF-5 and the 40S subunit-bound eIF-2 is critical for the hydrolysis of GTP

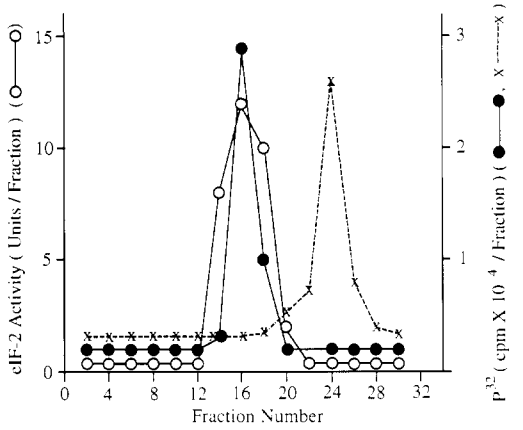

FIGURE 6: Specific association of exogenously added ^{32}P-labeled recombinant eIF-5 with eIF-2 present in a crude rabbit reticulocyte initiation factor preparation. A reaction mixture (400 μL) containing buffer A (see legend to Figure 5), 5% glycerol, 100 ng of [^{32}P]eIF-5 (100 000 cpm), and 200 μg of a partially purified phosphocellulose eIF-2/eIF-5 preparation, prepared as described under Experimental Procedures, was centrifuged as outlined in the legend to Figure 3. In a parallel tube, a similar reaction mixture containing ^{32}P-labeled eIF-5 but without partially purified eIF-2/eIF-5 preparation was sedimented. Following centrifugation, fractions of 0.35 mL were collected from the bottom of each tube and aliquots from each fraction counted for ^{32}P radioactivity. In addition, aliquots from each fraction obtained from the reaction mixture containing the eIF-2/eIF-5 preparation were also assayed for eIF-2 activity. In reaction mixtures containing the crude eIF-2/eIF-5 preparation, (●) represents [^{32}P]-eIF-5 while (○) represents eIF-2 activity. In reaction mixtures containing [^{32}P]eIF-5 alone, (×) represents [^{32}P]eIF-5.

bound to the 40S initiation complex. Such an interaction between eIF-5 and eIF-2 is directly demonstrated in the present work.

It is important to compare the mechanism of GTP hydrolysis in prokaryotic and eukaryotic systems. In prokaryotes, the initiation factor IF-2 alone is responsible both for the binding of the initiator fMet-tRNA$_f$ and GTP to the 30S ribosomal subunit to form the 30S initiation complex (30S·mRNA·fMet-tRNA$_f$IF-2·GTP·IF-1) and also for the subsequent hydrolysis of 30S subunit-bound GTP following addition of 50S ribosomal subunits [reviewed in Maitra *et al.* (1982)]. It appears, therefore, that in the eukaryotic system, the function of prokaryotic IF-2 is shared between the two proteins, eIF-2 and eIF-5. While eIF-2 is directly responsible for binding Met-tRNA$_f$ and GTP to the 40S ribosomal subunit, interaction of eIF-5 with 40S subunit-bound Met-tRNA$_f$eIF-2·GTP is essential for hydrolysis of GTP. It is likely that the increase in complexity of the mode of GTP hydrolysis in eukaryotic systems reflects the need for more intricate translational regulation in such systems.

Finally, it is also interesting to note that in addition to forming a specific complex with eIF-5, eIF-2 also forms specific complexes with eIF-2B (Siekierka *et al.*, 1982, 1984; Konieczny & Safer, 1983; Amesz *et al.*, 1979; Matts *et al.*, 1983) and 60S ribosomal subunits (Chakrabarti & Maitra, 1992; Thomas *et al.*, 1984; Gross *et al.*, 1985). All three initiation components, eIF-5, eIF-2B, and 60S ribosomal subunits, are known to be involved in the recycling of eIF-2 in the initiation reaction. In view of the well-established fact that a major point of translational control of gene expression occurs at the level of catalytic recycling of eIF-2 in initiation reactions (Hershey, 1991; Kozak, 1992), it is likely that protein–protein interactions which result in the sequestration of factors involved in the recycling of eIF-2 play an important role in the mechanism and regulation of translation initiation.

REFERENCES

Amesz, H., Goumans, H., Haubrich-Morree, T., Voorma, H. O., & Benne, R. (1979) *Eur. J. Biochem. 98*, 513–520.

Benne, R., Brown-Luedi, M. L., & Hershey, J. W. B. (1978) *J. Biol. Chem. 253*, 3070–3077.

Bradford, M. M. (1976) *Anal. Biochem. 75*, 248–254.

Chakrabarti, A., & Maitra, U. (1991) *J. Biol. Chem. 266*, 14039–14045.

Chakrabarti, A., & Maitra, U. (1992) *J. Biol. Chem. 267*, 12964–12972.

Chakravarti, D., & Maiti, T., Maitra, U. (1993) *J. Biol. Chem. 268*, 5754–5762.

Chevesich, J., Chaudhuri, J., & Maitra, U. (1993) *J. Biol. Chem. 268*, 20659–20667.

Conway, T. W., & Lipmann, F. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 1462–1469.

Das, K., Chevesich, J., & Maitra, U. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3058–3062.

Dubnoff, J. S., & Maitra, U. (1972) *J. Biol. Chem. 247*, 2876–2883.

Ghosh, S., Chevesich, J., & Maitra, U. (1989) *J. Biol. Chem. 264*, 5134–5140.

Gross, M., Redman, R., & Kaplansky, D. A. (1985) *J. Biol. Chem. 260*, 9491–9500.

Hathaway, G. M., Lundak, T. S., Tahara, S. M., & Traugh, J. A. (1979) *Methods Enzymol. 60*, 495–511.

Hershey, J. W. B. (1991) *Annu. Rev. Biochem. 60*, 717–755.

Konieczny, A., & Safer, B. (1983) *J. Biol. Chem. 258*, 3402–3408.

Kozak, M. (1983) *Microbiol. Rev. 47*, 1–45.

Kozak, M. (1992) *Annu. Rev. Cell Biol. 8*, 197–225.

Maitra, U., Stringer, E. A., & Chaudhuri, A. (1982) *Annu. Rev. Biochem. 51*, 869–900.

Matts, R. L., Levin, D. H., & London, I. M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2559–2563.

Merrick, W. C. (1992) *Microbiol. Rev. 56*, 291–315.

Merrick, W. C., Kemper, W. M., & Anderson, W. F. (1975) *J. Biol. Chem. 250*, 5556–5562.

Peterson, D. T., Safer, B., & Merrick, W. C. (1979) *J. Biol. Chem. 254*, 7730–7735.

Raychaudhuri, P., Chaudhuri, A., & Maitra, U. (1985a) *J. Biol. Chem. 260*, 2131–2139.

Raychaudhuri, P., Chaudhuri, A., & Maitra, U. (1985b) *J. Biol. Chem. 260*, 2140–2145.

Raychaudhuri, P., Chevesich, J., Ghosh, S., & Maitra, U. (1987) *J. Biol. Chem. 262*, 14222–14227.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Plainview, NY.

Schreier, M. H., Erni, B., & Staehelin, T. (1977) *J. Mol. Biol. 116*, 727–753.

Siekierka, J., Mauser, L., & Ochoa, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2537–2540.

Siekierka, J., Manne, V., & Ochoa, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 352–356.

Stringer, E. A., Chaudhuri, A., & Maitra, U. (1979) *J. Biol. Chem. 254*, 6845–6848.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60–89.

Thomas, N. S. B., Matts, R. L., Petryshyn, R., & London, I. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6998–7002.

Trachsel, H., & Staehelin, T. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 204–208.