Purification and Characterization of Initiation Factors IF-E4 and IF-E6 from Rabbit Reticulocytes*

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Initiation factors IF-E4 and IF-E6, which stimulate the synthesis of globin in vitro, have been isolated and purified from rabbit reticulocyte lysates. Crude initiation factors from the high salt ribosomal extract were separated by ammonium sulfate precipitation into Fraction A (0 to 40% saturation) and Fraction BC (40 to 70% saturation). Fraction BC was fractionated by chromatography on Sephadex G-100 into three fractions containing initiation factors IF-E2 plus IF-E5, IF-E4, and IF-E1, respectively. Initiation factor IF-E4 was further purified by column chromatography on DEAE-cellulose. It has a molecular weight of 40,000 and was obtained 80 to 90% pure. Initiation factor IF-E6 was purified from Fraction A by sucrose gradient centrifugation and column chromatography on DEAE-cellulose and phosphocellulose. Its molecular weight is 80,000 and the preparation was about 80% pure. Each factor was labeled with C-methyl groups by reductive alkylation without significantly altering the initiation factor activities as assayed by a protein synthesis system. It was shown by two-dimensional urea/sodium dodecyl sulfate-polyacrylamide gel electrophoresis that initiation factor IF-E6 does not correspond to the polypeptides of any other initiation factor or to any proteins present in ribosomes washed with high salt. However, initiation factor IF-E4 migrated coincident with polypeptide 5a, a minor component of initiation factor IF-E3.

Numerous initiation factors have been isolated from high salt extracts of ribosomes from rabbit reticulocytes. Schreier and Staehelin (1) and Staehelin et al. (2) have described six such factors, while Merrick and co-workers have isolated seven (3-7). Purification of each of the factors to homogeneity is helpful in order to elucidate the mechanism of action of the initiation factors and to demonstrate possible translational controls operating on the initiation process. We have previously described the purification of two factors, IF-E2' (8) and IF-E3 (9). IF-E2 is composed of three polypeptides and forms a ternary complex with Met-tRNA, and GTP. IF-E3 is a large multiprotein complex with 9 to 11 polypeptides. Both factors are required for protein synthesis in a system dependent on globin mRNA. We report here the purification and characterization of two additional initiation factors, IF-E4 and IF-E6, which are also required for optimal protein synthesis. IF-E4 and IF-E6 correspond closely to IF-E4 and IF-E6 reported previously by Staehelin et al. (2) and to IF-M4 and IF-M3, described by Merrick and co-workers (5, 6).

EXPERIMENTAL PROCEDURES

Materials — Rabbit reticulocyte globin mRNA and liver tRNA, and rat liver ribosomal subunits and pH 5 enzyme fraction were prepared as described previously (8, 9). Biochemical compounds were obtained as follows: creatine phosphokinase from Sigma Chemical Co.; [3H]methionine and [14C]formaldehyde from New England Nuclear; GTP, ATP, and creatine phosphate from Calbiochem; dithioerythritol from Pierce Chemical Co.; 2-mercaptoethanol from British Drug House; and acrylamide and bisacrylamide from Eastman. All other chemicals were reagent grade. Sephadex G-100 (Pharmacia), DEAE-cellulose (Whatman DE-32), and phosphocellulose (Whatman P-11) were pretreated according to the manufacturers' directions.

Buffers — Buffer A contained 20 mM Tris/Cl, pH 7.6, 7 mM 2-mercaptoethanol, and 5% glycerol. Buffer B contained 20 mM potassium phosphate, pH 7.2, 7 mM 2-mercaptoethanol, and 5% glycerol.

Initiation Factors — Crude initiation factors were isolated from rabbit reticulocyte lysates as described by Schreier and Staehelin (10). For assay ingredients, a small portion of the high salt ribosomal extract was treated with ammonium sulfate to yield Fraction A (0 to 40% saturation), Fraction B (40 to 70% saturation), and Fraction C (60 to 70% saturation). Fractions A, B, and C were dialyzed against 20 mM Tris/HCl, pH 7.6, 7 mM 2-mercaptoethanol, 5% glycerol (Buffer A), and 100 mM KCl, clarified by centrifugation and used as ingredients in the assays described below. For the preparative procedures, batches of the high salt ribosomal extract obtained from 30 rabbits were treated with ammonium sulfate to yield Fraction A (0 to 40% saturation) and Fraction BC (40 to 70% saturation). Fraction A was used as the source of IF-E3 and IF-E6. Fraction BC served as the source of IF-E4 and IF-E5.

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source of IF-E1, IF-E2, IF-E4, and IF-E5. Purified IF-E3 was prepared as described previously (9).

**Assays for Initiation Factors** - IF-E2 activity was assayed as described previously (8) by measuring ternary complex formation between IF-E2, [3H]Met-tRNA, and GTP. The other initiation factor activities were measured in a system for protein synthesis developed by Schreier and Staehelin (10). Each assay mixture of 100 μl contained: 20 mM Tris/HCl, pH 7.6, 4 mM magnesium acetate, 70 to 80 mM KCl, 10 mM 2-mercaptoethanol, 0.03 mM each of 19 nonradioactive amino acids and [3H]leucine, specific activity 500 Ci/mmol, 25 mM creatine phosphate, 1 mM ATP, 0.14 mM GTP, 4 units of creatine phosphokinase, 0.2 A₅₅₀ unit of 40 S ribosomal subunits, 0.5 A₅₅₀ unit of 60 S ribosomal subunits, 0.05 A₅₅₀ unit of globin mRNA, 5 μl of ϕ80 enzyme fraction, 0.5 A₅₅₀ unit of rabbit liver tRNA, and initiation factor fractions as follows. The IF-E4 assay utilized 20 μg of Fraction A, 20 μg of Fraction B, and 0.12 μg of IF-E4 purified by phosphocellulose chromatography. The IF-E6 assay utilized: 10 μg of pure IF-E3, 15 μg of Fraction B, and 15 μg of Fraction C. The IF-E5 assay utilized: 50 μg of Fraction A, 20 μg of Fraction B, and 1.7 μg of protein containing IF-E4 from a Sephadex G-100 column (see Fig. 1). The volume of each factor assayed is given in the figure or table legends.

**Results**

**Fractionation of Crude Initiation Factors in Fraction BC**

The high salt ribosomal extract was first fractionated by ammonium sulfate precipitation into the A and BC fractions as described under "Experimental Procedures." The BC fraction obtained from 60 rabbits was dissolved in Buffer A containing 100 mM KCl and clarified by centrifugation. The solution was added without prior dialysis to remove ammonium sulfate to a Sephadex G-100 column equilibrated in Buffer A and 100 mM KCl. The protein was eluted with the same buffer; fractions of 3.1 ml were collected every 40 min and 5-μl aliquots were assayed for IF-E2, IF-E4, and IF-E5 activities as described under "Experimental Procedures." Protein content was determined by measuring absorbance at 280 nm in a Gilford 2400 spectrophotometer. A△A, IF-E1 activity; □, IF-E2 activity; ●, IF-E4 activity. B, DEAE-cellulose. The pooled fractions containing IF-E4 from the Sephadex G-100 column (65 mg in 23 ml) were passed through a column (0.9 x 280 cm) of DEAE-cellulose equilibrated in Buffer A and 100 mM KCl. Adsorbed protein was eluted with a 150-ml linear salt gradient containing 100 to 400 mM KCl in Buffer A, and 1.6-ml fractions were collected every 20 min. Protein concentration was determined as in A (□), aliquots of 5 μl were assayed for IF-E4 activity as described under "Experimental Procedures."

Stimulatory activity for globin synthesis was also found in those fractions from the Sephadex G-100 column corresponding to proteins of low molecular weight (Fractions 27 to 45). The active component(s) was further purified by salt gradient elution from a phosphocellulose column. A protein was obtained with a molecular weight of 15,000 which stimulated protein synthesis about 10-fold in the IF-E1 assay system described under "Experimental Procedures." This protein most likely corresponds to IF-E1 described previously by Staehelin et al. (2). Details of the purification of this initiation factor will be given elsewhere.

**Purification of IF-E4** - IF-E4 obtained from Sephadex chromatography was further purified by column chromatography on DEAE-cellulose. The pooled IF-E4 fractions (29 to 36) were passed through a column of DEAE-cellulose equilibrated in Buffer A and 100 mM KCl. Adsorbed protein was eluted with a salt gradient and the fractions were analyzed for IF-E4 activity and by SDS-polyacrylamide gel electrophoresis. The results are shown in Fig. 1B and details of the procedures are given in the figure legend. IF-E4 activity was eluted at approximately 210 mM KCl. The fractions containing IF-E4...
Purification and Characterization of IF-E4 and IF-E6

The values cited for IF-E4 are derived from a typical preparation obtained from the blood of 60 anemic rabbits; those for IF-E6 were obtained from a preparation of 180 rabbits. Protein was measured by absorbance at 280 nm, and 1 unit is defined as the amount of protein in 1 ml of solution which has an A_{280} of 1 in a 1-cm cell. Activities were measured as described under "Experimental Procedures"; 1 activity unit refers to the ability to stimulate the incorporation of 1 nmol of [3H]leucine into protein under standard assay conditions. Specific activity is defined as activity units divided by A_{280} units.

Table I

<table>
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<tr>
<th>Factor</th>
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<th>Protein</th>
<th>Activity</th>
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<th>Recovery</th>
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<tr>
<td>IF-E4</td>
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<td>2000</td>
<td>units</td>
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<tr>
<td></td>
<td>Ammonium sulfate (BC)</td>
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<tr>
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<td>28.7</td>
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<td>11.4</td>
<td>9.5</td>
<td>100</td>
</tr>
<tr>
<td>IF-E6</td>
<td>High salt extract</td>
<td>6000</td>
<td>units</td>
<td>units/A_{280}</td>
<td>%</td>
</tr>
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<td>Ammonium sulfate (A)</td>
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<td>Sucrose gradient</td>
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<td>100</td>
</tr>
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<td></td>
<td>Phosphocellulose</td>
<td>2.4</td>
<td>18.7</td>
<td>7.80</td>
<td>36</td>
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</table>

(Fractions 42 to 52) were pooled and the protein was concentrated to 1.9 ml by ultrafiltration (Amicon PM-10 filter). The yield was 1.2 mg and the purity as determined by densitometric tracings of stained SDS-polyacrylamide gels was 80 to 90% as shown in Fig. 3. IF-E4 preparations at this stage of purification sometimes contained a component which partially inhibited [3H]Met-tRNA binding to 40 S subunits in the presence of the other initiation factors. The contaminant was removed by passing the preparation through a small phosphocellulose column (0.9 x 18 cm) equilibrated with Buffer B and 100 mM KCl. IF-E4 did not adsorb to the column and was recovered and concentrated as described above. A summary of the amounts of protein and activities obtained at each step of the purification is shown in Table I.

Purification of IF-E6 - The ammonium sulfate Fraction A (0 to 40% saturation) obtained as described under "Experimental Procedures" contained both IF-E3 and IF-E6. The A fractions obtained from 180 rabbits were pooled and the two factors were separated by centrifugation in sucrose gradients as described previously (9). The top fractions of the gradients contained the IF-E6 activity and were pooled. IF-E6 was purified further by column chromatography on DEAE-cellulose. The resulting absorbance and activity profiles are shown in Fig. 2A; procedures are described in the figure legend. IF-E6 was eluted at approximately 160 mM KCl and the active fractions (46 to 72) were combined. The solution was diluted with 1 volume of Buffer B to reduce the salt concentration and IF-E6 was further purified by phosphocellulose column chromatography as described in the legend to Fig. 2B. IF-E6 activity was eluted at approximately 300 mM KCl and corresponded to the major protein peak. Active fractions (100 to 120) were combined and concentrated to 4 ml by ultrafiltration (Amicon PM-10 filter) to yield 2.4 mg of protein. The preparation was about 80% pure as calculated from the densitometric tracing of the SDS-polyacrylamide gel shown in Fig. 3. The amounts of protein and factor activity following each purification step are given in Table I. From the sucrose gradient step, IF-E6 was purified 18-fold and was obtained in 36% yield.

Physical and Biological Characterization of IF-E4 and IF-E6 - Both initiation factors show one major protein component when analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Molecular weights were determined by their migration rates relative to protein standards according to the method of Weber and Osborn (11). Values of 49,000 for IF-E4 and 80,000 for IF-E6 were obtained (data not shown). Analysis of IF-E4 and IF-E6 by two-dimensional polyacrylamide gel electrophoresis (described in Fig. 6 below) revealed a single major protein component for each. Both factors are required for optimal protein synthesis in a system dependent

Fig. 2. Column chromatography of IF-E6 on DEAE-cellulose and phosphocellulose. A, DEAE-cellulose. Fractions containing IF-E6 from the sucrose gradient (approximately 120 mg of protein in 12 ml) were added to a column of DEAE-cellulose (2 x 25 cm) equilibrated in Buffer A and 100 mM KCl. Adsorbed protein was eluted with a 300-ml linear salt gradient containing 100 to 300 mM KCl in Buffer A; 3-ml fractions were collected every 15 min. Protein concentration was determined by absorbance at 280 nm (-----) and aliquots of 5 μl were assayed for IF-E6 activity (•--•) as described under "Experimental Procedures." B, Phosphocellulose. The diluted fractions from the DAE-cellulose column (10 mg of protein in 160 ml) were added to a column of phosphocellulose (1.4 x 20 cm) equilibrated in Buffer B and 100 mM KCl. The adsorbed protein was eluted with a 300-ml linear salt gradient, 100 to 500 mM KCl in Buffer B, and fractions of 1.5 ml were collected every 15 min. Protein (-----) and IF-E6 activity (•--•) were determined as described in A.
on globin mRNA. As shown in Fig. 4, purified IF-E4 causes a 4- to 5-fold stimulation of protein synthesis; purified IF-E6 stimulates 3- to 4-fold.

Radioactive Labeling by Reductive Alkylation without Significant Loss of Biological Activity—After passage through phosphocellulose columns, IF-E4 and IF-E6 were each subjected to reductive alkylation as described under "Experimental Procedures." The resulting specific radioactivities were approximately 10,000 cpm/µg of protein for 14CH3-IF-E4 and 7,500 cpm/µg of protein for 11CH3-IF-E6, which corresponds to about five 14CH3 groups/factor molecule. SDS polyacrylamide gel electrophoresis of the labeled factors showed that approximately 85% of the radioactivity present in the 14CH3-IF-E4 and 75% of the 11CH3-IF-E6 migrated to positions that corresponded to the unlabeled factors (Fig. 5). 14CH3-IF-E4 and 11CH3-IF-E6 were assayed as described in Fig. 4 under conditions of linear response of the factors and compared to unlabeled factors. As shown in Table II, the factor activity of labeled IF-E4 and IF-E6 was 75 to 80% of the untreated factors.

Comparison with Other Initiation Factor Polypeptides and Ribosomal Proteins—The molecular weight of IF-E6 is not similar to that of any of the polypeptide components of IF-E2, IF-E3, or other initiation factors. On the other hand, the molecular weight of IF-E4 is similar to polypeptide 2 (53,000) of IF-E2 (8) and to polypeptides 5, 5a, and 6 (45,000 to 50,000) of IF-E3 (9). In order to investigate possible identities, we compared 14CH3-IF-E4 and 11CH3-IF-E6 with the polypeptides of IF-E2 and IF-E3 by co-electrophoresis in two-dimensional urea/SDS-polyacrylamide gels. As shown in Fig. 6, 14CH3-IF-E4 does not correspond to any of the polypeptides of IF-E2 or IF-E3. However, 11CH3-IF-E4 coincides with polypeptide 5a of IF-E3. Polypeptide 5a was present in variable amounts in IF-E3 preparations and was not part of the IF-E3 complex following binding to 40 S ribosomal subunits (9).

The possibility that IF-E4 or IF-E6 may correspond to ribosomal proteins was also tested by co-electrophoresis of 11CH3-IF-E4 and 11CH3-IF-E6 with ribosomal proteins from E. coli.

**Table II**

Comparison of activity of labeled and unlabeled factors in protein synthesis

The protein synthesis assays for IF-E4 and IF-E6 are described under "Experimental Procedures." In Experiment I, 0.6 µg of IF-E4 or 14CH3-IF-E4 were added; in Experiment II, 0.3 µg of IF-E6 or 11CH3-IF-E6. Values obtained in control experiments performed in the absence of either IF-E4 or IF-E6 have been subtracted.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incubation Time</th>
<th>14HLeucine incorporated (µg)</th>
<th>Inhibition by labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>µmol</td>
<td>IF-E4</td>
</tr>
<tr>
<td>I</td>
<td>10</td>
<td>2.37</td>
<td>1.78</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.20</td>
<td>3.52</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5.45</td>
<td>4.18</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>0.95</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>2.12</td>
<td>1.71</td>
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<td>30</td>
<td>3.16</td>
<td>2.41</td>
</tr>
</tbody>
</table>
IF-E4 and \(^{14}C\)-IF-E6 and a total protein extract of 60 S ribosomal subunits prepared from rabbit reticulocyte ribosomes washed in high salt buffer (13) None of the radioactive factors coincided with any of the stained ribosomal proteins (data not shown). Since there are no 40 S ribosomal proteins larger than 45,000 daltons (14), we conclude that neither IF-E4 or IF-E6 is a ribosomal protein.

**DISCUSSION**

A procedure has been developed for the separation of crude initiation factors from rabbit reticulocyte and for their subsequent purification to near homogeneity. Two fractions were obtained by ammonium sulfate precipitation and each was subjected to a procedure which fractionates proteins on the basis of size. The ammonium sulfate A fraction (0 to 40% saturation) was subjected to sucrose density centrifugation to separate IF-E3 and IF-E6 activities. Each factor was subsequently purified by ion exchange chromatography on phosphocellulose and DEAE-cellulose. The ammonium sulfate BC fraction (40 to 70% saturation) was subjected to molecular sieve chromatography with Sephadex G-100, which separated the factors into three fractions, one containing IF-E2 and IF-E5, one containing IF-E4, and a third containing IF-E1 and possibly other factors of low molecular weight. Purified IF-E4 was obtained simply by chromatography on DEAE-cellulose. The procedures described here resulted in relatively good yields of IF-E4 and IF-E6, which were 80 to 90% pure.

IF-E4 and IF-E6 are both single polypeptide chains with molecular weights of 49,000 and 80,000, respectively. Polyacrylamide gel electrophoresis on a two-dimensional system employing urea and SDS buffers did not reveal additional major components in the preparations (Fig. 5). Based on its molecular weight and behavior on DEAE-cellulose and phosphocellulose ion exchange columns, the IF-E4 purified here very likely corresponds to IF-E4 (\(M_r = 50,000\)) described by Staehein's group (1, 2), to IF-M4 (\(M_r = 48,000\)) reported by Merrick and co-workers (6), and to IF-EMC described by Weigle and Smith (15). Similarly, IF-E6 appears to correspond to the IF-E6 of Staehein et al. (2) and to IF-M3 which was purified by Frick and Anderson (5) and analyzed by SDS-polyacrylamide gel electrophoresis by Safer et al. (6). In agreement with the Staehein and Merrick groups (2, 6), we find that both factors are required for the translation of globin mRNA. In the assays employed here, about 1 \(\mu\)g of each factor was required to maximally stimulate globin synthesis (Fig. 4).

IF-E6 does not correspond to any of the known initiation factor protein or to any of the protein components of ribosomes washed in high salt buffer. However, IF-E4 corresponds to polypeptide 5a of IF-E3, the large multiprotein complex previously described and characterized in this laboratory (9). It has been suggested that IF-E3 may be a complex containing many initiation factors which also have been purified separately. Safer et al. (6) reported that IF-M5, which corresponds closely to IF-E3 (9), also contains small amounts of other factor proteins, i.e. IF-M2A (IF-E5), IF-M3 (IF-E6), a component of IF-MP (IF-E2), and elongation factor EF-2, in addition to the large set of unique components. Co-purification of these factors with IF-E3 suggests that they are bound to the IF-E3 complex and that they function together as a very large, but labile, complex during the initiation process. However, when the IF-E3 complex binds to the 40 S ribosomal subunit, the minor components and polypeptide 5a (IF-E4) are no longer part of the ribosomal subunit initiation factor complex. Only nine polypeptides of IF-E3, none of which corresponds to any other of the known initiation factors, are components of this complex (9). This suggests that proteins like IF-E4 and IF-E6 are not integral parts of IF-E3, but on isolation, may be bound to the factor complex in varying amounts. Furthermore, binding of radioactively labeled IF-E4 and IF-E6 to 40 S ribosomal subunits is highly dependent on the presence of IF-E2 and IF-E3 in the 40 S complexes. These results, taken with the facts discussed above, suggest that IF-E3 and the other factors may bind contiguously on the surface of the 40 S ribosomal subunit. Thus, each of the factor proteins may interact directly with a part of the IF-E3 complex as well as with the ribosomal subunit. The possibility that interactions occur both in the presence and in the absence of ribosomal subunits may be tested directly with cross-linking reagents, such as have been used to study initiation factor-ribosome interactions in bacteria (16, 17).

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Purification and characterization of initiation factors IF-E4 and IF-E6 from rabbit reticulocytes.
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