Purification and Characterization of Homogeneous Protein Synthesis Initiation Factor M1 from Rabbit Reticulocytes

(Received for publication, May 8, 1974)

WILLIAM C. MERRICK AND W. FRENCH ANDERSON
From the Molecular Hematology Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

An eight-step procedure has been devised for the preparation of homogeneous rabbit reticulocyte IF-M1. Molecular weight determinations based on IF-M1 activity (gel filtration and sucrose density gradient sedimentation) and based on IF-M1 protein (low speed equilibrium sedimentation and sodium dodecyl sulfate gel electrophoresis) indicate that IF-M1 is active as a single polypeptide chain of 55,000 molecular weight. The amino acid composition of IF-M1 has been determined. There appears to be no unique features in the amino acid composition of IF-M1, except perhaps an elevated proline content (6.9 mol %).

The catalytic properties of purified IF-M1 were similar to those previously reported by this laboratory for crude preparations of IF-M1. The sensitivity of IF-M1 activity to N-ethylmaleimide and heat (45°C) inactivation was tested in two model reactions requiring minimal complementary factors: (a) AUG-directed fMet-tRNA binding to ribosomes; and (b) poly(U)-directed polyphenylalanine synthesis at 4 mM Mg2+ (IF-M2A, IF-M2B, EF-1, and EF-2 also required). IF-M1 activity proved to be sensitive to both N-ethylmaleimide and temperature (45°C). In addition, a contaminant of partially purified IF-M1 preparations has been found which is capable of fMet-tRNA binding but is inactive in poly(U)-directed polyphenylalanine synthesis at low Mg2+ concentration.

EXPERIMENTAL PROCEDURE

Materials

Dithiothreitol, ATP, and GTP were purchased from Calbiochem. N-ethylmaleimide was obtained from Sigma Chemical Co. Radioactive amino acids were from Schwarz-Mann. Crude Escherichia coli B tRNA and a cell paste of E. coli W were purchased from General Biochemicals. Purified E. coli tRNAphe, poly(U), and ApUpG were obtained from Miles Laboratories, Inc. All other materials used were of reagent grade quality. The proteins used as molecular weight standards for sodium dodecyl sulfate gel electrophoresis and sucrose density gradients were ovalbumin, a-galactosidase and DNase I (Worthington Biochemical Corp.), bovine serum albumin and bovine catalase (Schwarz-Mann), rabbit muscle aldolase (Pharmacia Fine Chemicals, Inc.), and rabbit muscle pyruvate kinase (Calbiochem).

Preparation of IF-M1

Step 1: 0.5 M KCl Ribosomal Wash—Rabbit reticulocyte lysate and 0.5 M KCl ribosomal wash fraction were prepared as reported previously (18, 19). The lysate from 600 rabbits was used to generate the starting material of 24,300 mg of ribosomal wash protein.

Step 2: (NH4)2SO4 Precipitation—The 0.5 M KCl ribosomal wash fraction was concentrated by (NH4)2SO4 precipitation (70%) using neutralized (NH4)2SO4 (a 50:1 w/w mixture of (NH4)2SO4 and (NH4)2CO3). The (NH4)2SO4 precipitation and all subsequent steps were performed at 2-4°C unless stated otherwise. The precipitated protein was collected by centrifugation and then was dissolved in and dialyzed overnight against cold Buffer A (20 mM

1 The abbreviations used are: IF, initiation factor; EF, elongation factor; Heps, N-2-hydroxylethylpiperazine-N'-2-ethanesulfonic acid; fMet, N-formylmethionine; NEM, N-ethylmaleimide; poly(U) assay, poly(U)-directed polyphenylalanine synthesis at 4 mM Mg2+.
Tris-HCl (pH 7.5)-1 mm dithiothreitol-0.1 mm EDTA) containing 50 mM KCl.

**Step 7: DEAE-cellulose Chromatography**—Approximately 100 ml of concentrated 0.5 mM KCl ribosomal wash fraction (20 mg per ml) were applied to a DEAE-cellulose column (2.5 X 40 cm; Whatman DE52) equilibrated with Buffer A containing 50 mM KCl. After the sample had been applied, the column was washed with Buffer A containing 50 mM KCl until no additional ultraviolet absorbing material was eluted from the column. The tubes containing the protein which was not adsorbed to the DEAE-cellulose column (IF-M1) were pooled. Protein which was adsorbed to the column was used for the purification of the other initiation factors (20, 21).

**Step 4: (NH₄)₂SO₄ Fractionation**—The protein from Step 3 was precipitated between 35 and 60% neutralized (NH₄)₂SO₄, collected by centrifugation, and then dissolved in and dialyzed overnight against Buffer A containing 100 mM KCl.

**Step 5: Sephadex G-200 Chromatography**—The 35 to 60% (NH₄)₂SO₄-fractionated IF-M1 (10 ml; 40 mg per ml) was applied to a Sephadex G-200 column (2.5 X 90 cm) equilibrated with Buffer A containing 100 mM KCl. IF-M1 was eluted from the column with the same buffer; 5-ml fractions were collected every 20 min. IF-M1 activity eluted between 1/2 and 2 void volumes (55% of the bed volume). The pooled material was concentrated by (NH₄)₂SO₄ precipitation (80%), dialyzed against Buffer A containing 100 mM KCl, and stored in aliquots in liquid nitrogen at a concentration of 0.13 mg of protein per ml (approximately 130 mg of total protein).

**Step 6: CM-cellulose Chromatography**—IF-M1, obtained from Sephadex G-200 chromatography, was chromatographed on a carboxymethylcellulose column (0.9 X 50 cm; Whatman CM52) equilibrated with Buffer B (20 mM Hepes-KOH (pH 6.2)-1 mm dithiothreitol-0.1 mm EDTA) containing 50 mM KCl. To adjust the salt concentration and pH to that of 50-mM KCl Buffer B, the IF-M1 (in 100 mM KCl Buffer A) was diluted with an equal volume of cold Buffer B with stirring. The pH of the diluted solution was adjusted to pH 6.2 by the dropwise addition of 1 mM potassium acetate, pH 5.0. The diluted sample was applied to the CM-cellulose column at a flow rate of 12 ml per hour. The column was washed with 40 ml of Buffer B containing 50 mM KCl. IF-M1 was eluted with a linear salt gradient (300 X 400 ml) from 50 to 300 mM KCl. IF-M1 was eluted with a linear salt gradient (300 X 400 ml) from 50 to 300 mM KCl. IF-M1 activity was eluted from the column with a linear salt gradient (400 X 400 ml) from 200 to 600 mM KCl (in Buffer C). Five-milliliter fractions were collected every 20 min. IF-M1 activity eluted at approximately 450 mM KCl (see Fig. 1). Tubes containing IF-M1 activity were pooled and concentrated to 7 ml by ultrafiltration (Amicon, UM-2 membrane). The concentrated IF-M1 was stored in aliquots in liquid nitrogen at a concentration of 0.5 mg of protein per ml.

**Step 8: Sephadex G-100 Chromatography**—The final purification step of IF-M1 was chromatography on a Sephadex G-100 column (1.6 X 87 cm). Six milliliters of IF-M1 (0.5 mg per ml) were applied to a Sephadex G-100 column which had been equilibrated with Buffer A containing 100 mM KCl. Protein was eluted at a flow rate of 10 ml per hour and 2-ml fractions were collected (see Fig. 2). Tubes containing IF-M1 activity were pooled as indicated in Fig. 2 and were concentrated to 0.6 mg of protein per ml by ultrafiltration (Amicon, UM-2 membrane). The concentrated preparation was clarified by centrifugation (6000 X g for 10 min) and was stored in aliquots in liquid nitrogen. The purification procedure for IF-M1 is summarized in Table I.

**Preparation of Ribosomes**

Ribosomes obtained from the high-speed centrifugation of rabbit reticulocyte lysate were dissolved in standard sucrose (250 mM sucrose-1 mm dithiothreitol-0.1 mM EDTA, pH 7.0) at a concentration of 250 A₂₅₀ units per ml. The ribosome solution was made 0.5 M KCl by the gradual addition of solid KCl and then was stirred slowly for 30 min at 4°C. The 0.5 M KCl ribosome solution was layered (6 ml per tube) over a 1.0 M sucrose cushion (3 ml per tube): 1.0 M sucrose; 0.5 M KCl; 20 mM Tris-HCl, pH 7.5; 2 mm MgCl₂; 0.1 mM EDTA; 1 mm dithiothreitol) and then was centrifuged for 4 hours at 60,000 rpm (Spinco TI 75 rotor). The supernatant was poured off and the free surface of the ribosome pellet was rinsed with standard sucrose. The pellets were dissolved in standard sucrose and were clarified by centrifugation (1000 X g for 10 min). Aliquots were stored in liquid nitrogen at a concentration of 380 A₁₆₀ units per ml. These ribosomes were used in place of salt-washed ribosomes because better dependencies upon initiation and elongation factors were obtained when using...
the poly(U)-directed polyphenylalanine synthesis assay. Ribosomes prepared in this manner consist primarily of monosomes and subunits.

**Preparation of Aminoacyl-tRNA**

[14C]Phenylalanine (E. coli) (specific activity 450 mCi per mmol) was prepared as described previously (22). [3H]Met-tRNA\textsubscript{f} (specific activity 2300 mCi per mmol) was prepared using rabbit reticulocyte tRNA\textsubscript{f}et and a crude E. coli aminoacyl-tRNA synthetase preparation (22).

**Assays**

IF-Ml activity was routinely tested using the poly(U)-directed polyphenylalanine synthesis assay at low (4 mM) Mg\textsuperscript{2+} concentration as described previously (19) except that salt-washed sucrose cushion ribosomes, as described above, were substituted for salt-washed ribosomes. The preparation of the complementary factors required for the assay (IF-M2A, IF-M2B, EF-1, and EF-2) has been reported (20, 23). Each of the complementary factors was used in saturating amounts (IF-M2A, 11.2 μg; IF-M2B, 10.5 μg; EF-1, 7.9 μg; and EF-2, 1.3 μg). For convenience, this assay will be referred to as the poly(U) assay in the remainder of this paper. AUG-directed fMet-tRNA\textsubscript{f} binding utilizing salt-washed ribosomes was performed as described previously (16).

**Reaction with N-Ethylmaleimide**

Samples to be tested for N-ethylmaleimide inactivation were incubated in 10 mM NEM for various lengths of time at room temperature. The NEM was neutralized then by the addition of dithiothreitol to a final concentration of 100 mM and was incubated at room temperature for 5 min. Controls included a 5-min incubation of 10 mM NEM and 100 mM dithiothreitol at room temperature followed by the addition of sample and continued incubation for the indicated time.

**Disc Gel Electrophoresis**

Disc gel electrophoresis was performed as described by Canaleo (24) using 6% acrylamide running gels and acetic buffers (stacking at pH 5.0, electrophoresis at pH 4.3). Protein samples were subjected to electrophoresis for approximately 1 hour at 4 mA per gel (gel tubes 5 mm inner diameter × 75 mm length). Staining of the gels (presoaked in cold 12.5% trichloroacetic acid) with Coomassie brilliant blue and destaining in acidic methanol were as described by Canaleo (94).

**Molecular Weight Determination by Sodium Dodecyl Sulfate Gel Electrophoresis**

The molecular weight of IF-Ml was estimated by its relative electrophoretic mobility in acrylamide gels containing sodium dodecyl sulfate as described by Weber and Osborn (25) with the following modifications. (a) The final gel concentration was 6% instead of 10%; and (b) the gel buffer and the electrophoresis buffer had one-half of the ionic strength. Gels were subjected to electrophoresis for 2 hours at 8 mA per gel. Under these conditions, tracking dye (dromophenol blue) migrated approximately 90 mm (gel tubes 5 mm inner diameter × 125 mm length). Staining (2 to 5 hours) and diffusion destaining (18 to 36 hours) were as described (25).

**Molecular Weight and Stokes Radius Determination by Gel Filtration**

A 200-μl sample containing IF-Ml (Step 5) and standard protein markers was applied to a column of Sephadex G-150 (0.9 × 60 cm) which had been equilibrated with Buffer A containing 100 mM KCl. Protein was eluted with the same buffer at a flow rate of 4 ml per hour; 0.5-ml fractions were collected. The elution position of standard molecular weight marker proteins was determined by absorbance at 280 nm with a Beckman DU-2 spectrophotometer. Molecular weights of the standard proteins were supplied by the manufacturer. Stokes radii were from published sources (26) or were determined from published D\textsubscript{so} values (27) by the equation $a = kT/6\pi ND$ (where $a$ is Stokes radius, $k$, Boltzmann constant; $T$, absolute temperature; $\eta$, viscosity of the medium; and $D$, diffusion coefficient).

**Sedimentation Equilibrium Centrifugation**

The $S$ value of IF-Ml (Step 5) was determined by the comparison of its rate of sedimentation in sucrose gradients to that of proteins of known $S$ values. Linear sucrose gradients from 5 to 20% sucrose (w/v) contained 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 100 mM KCl, and 0.1 mM EDTA. Samples, 250 μl each, containing IF-Ml or standard protein markers or both were layered over chilled sucrose gradients and were centrifuged for 43/2 hours at 65,000 rpm at 4° with a Beckman SW 65 rotor. The sucrose gradients were unloaded and monitored for absorbance at 280 nm, and 125-μl fractions were collected using an ISCO density gradient fractionator and a Gilford model 2000 spectrophotometer with a flow cell attachment. Aliquots (20 μl) were tested for IF-Ml activity using the poly(U) assay.

**Determination of $S_{20\text{w}}$**

IF-Ml (Step 8) was dialyzed at 4° overnight against 2 liters of 100 mM KCl, 10 mM Tris-HCl, pH 8.0 (20°), and 0.1 mM EDTA. N\textsubscript{2} was bubbled in the buffer during the entire dialysis period. The IF-Ml (0.6 ml, 0.6 mg of protein per ml) was clarified by centrifugation (4,000 × g for 10 min) and was stored in liquid nitrogen until used. Aliquots of the dialysis buffer were saved to be used as reference buffer. The $S_{20\text{w}}$ of IF-Ml was determined in a Beckman model E ultracentrifuge using an ultraviolet scanner (280 nm). The sedimentation run was performed at 48,000 rpm at 22.5° (zero time was taken as attainment of 32,000 rpm during acceleration; 48,000 rpm was obtained 8 min later). The data were analyzed as described by Chervenka (28).

**Sedimentation Equilibrium Centrifugation**

IF-Ml was diluted with dialysis buffer to a concentration of 0.2 mg of protein per ml and 0.2 ml of this solution was centrifuged for 72 hours at 9,000 rpm at 8° in a Beckman model E ultracentrifuge. Protein concentration in the sample cell was determined using the ultraviolet scanner (280 nm). Achievement of equilibrium was checked during the run and essentially was complete at 48 hours. Zero protein concentration was determined as follows. After scanning the sample cell (at 72 hours), the rotor was accelerated to 40,000 rpm to deplete the meniscus of protein; after centrifugation at 40,000 rpm for 4 hours, the rotor was decelerated to 9,000 rpm and then the sample cell was scanned to determine zero protein concentration (at the meniscus). Analysis of the
data was performed as described by Chervenka (28). The authors would like to thank Dr. Robert Simpson of the National Institute of Arthritis, Metabolism, and Digestive Diseases for kindly performing the ultracentrifugal analyses.

Amino Acid Composition

Samples for amino acid analysis were made 20% in acetic acid and then were chromatographed on a column of P-30 (0.9 X 55 cm) (Bio-Rad) which had been equilibrated with 20% acetic acid. The tubes containing the protein which was excluded from the column were pooled. The pooled fraction was diluted 1 to 4 with distilled H2O, frozen, and lyophilized to dryness. The purpose of the chromatography in 20% acetic acid was to remove any nonspecifically bound carbohydrate which might interfere with the hydrolysis.

Duplicate lyophilized samples were hydrolyzed under vacuum in 4 M methane sulfonic acid made 0.02% in tryptamine for either 22 or 72 hours as described by Liu (29). Following hydrolysis, cysteine residues were converted to S-sulfocysteine as described by Liu and Inglis (30). Amino acid analysis was performed on a Beckman model 120B amino acid analyzer. Values for tryptophan, serine, and threonine were extrapolated to zero time to correct for any decomposition during hydrolysis. Values for leucine, isoleucine, and valine were extrapolated to infinite time to allow for complete release. The authors would like to thank Dr. Richard Simpson of the National Institute of Child Health and Human Development for kindly performing the amino acid analyses.

RESULTS

Purification of IF-M1

The purification procedure for IF-M1 is given under "Experimental Procedures" and is summarized in Table I. Step 1 is taken as the ribosomal wash protein. Step 2 in the purification of IF-M1 is a 70% (NH4)2SO4 precipitation which concentrates the ribosomal wash protein and which also removes approximately 35% of the contaminating hemoglobin. Step 3, DEAE-

![Fig. 3](left). Polyacrylamide disc gel electrophoresis of IF-M1. Acid disc gel electrophoresis was performed as described under "Experimental Procedure." The samples were: A, 12 μg of Step 7 IF-M1; B, C, D, and E, 0.4, 1.2, 3.6, and 8.0 μg of Step 8 IF-M1, respectively.

![Fig. 4](right). Sodium dodecyl sulfate polyacrylamide gel electrophoresis of IF-M1. Electrophoresis was performed as described under "Experimental Procedure." Samples (from left to right) were 0.4, 1.2, and 8.0 μg of Step 8 IF-M1.
tions. Only for Steps 1 and 2 was there more than a 10% variance in different specific activity determinations.

**Physical Properties of Homogeneous IF-M1**

**Amino Acid Composition**—The amino acid composition of IF-M1 is given in Table II. There appears to be no unique characteristic about this composition except perhaps a slightly elevated proline content (6.9 mol %). The $\bar{v}$ of IF-M1 is 0.731 cm$^3$ per g when determined from the amino acid composition as described by Cohn and Edsall (31).

**Absorption Spectra**—IF-M1 (Step 8) was dialyzed overnight against 100 mM KCl, 10 mM Tris-HCl (pH 8.0), and 0.1 mM EDTA. The spectrum of IF-M1 was obtained using a Cary 15 recording spectrophotometer with dialysis buffer used as reference solution (see Fig. 5). The absorption maximum was at 278 nm.

**Sedimentation Velocity Centrifugation**—The dialyzed IF-M1 preparation was used for determination of $s_{20,w}$. Sedimentation was performed at 48,000 rpm at 22.5° and the sedimentation of IF-M1 was monitored with an ultraviolet scanner (280 nm). The $s_{20,w}$ was calculated at 25, 50, and 75% of the plateau absorbance value and all three values were 3.56. The plot of log $R$ versus time in Fig. 6 is for $R$ at 50% of the plateau absorbance. Correction for temperature and salts yield an $s_{20,w}$ value of 3.35.

**Sedimentation Equilibrium Centrifugation**—An IF-M1 molecular weight estimation using sedimentation equilibrium techniques was performed using dialyzed IF-M1 which had been diluted 1:3 with dialysis buffer (see “Absorption Spectra”). The sample was centrifuged for 72 hours at 9,000 rpm at 8°. Under these conditions, there was protein present from the meniscus to the bottom of the cell. After the cell was scanned (280 nm) at equilibrium, the rotor was accelerated to 40,000 rpm so as to deplete the meniscus of protein. Following the 4-hour centrifugation at 40,000 rpm, the rotor was decelerated to 9,000 rpm and the cell was scanned to establish the absorption level of zero protein concentration (at the meniscus). The plot of log $C$ versus $R^2$ yielded a slope of 0.1475 (Fig. 7). The $M(1 - \bar{v})$ value was 17,870 which corresponds to a molecular weight of 66,400 using a $\bar{v}$ of 0.731 cm$^3$ per g (determined from amino acid composition).

**Sodium Dodecyl Sulfate Gel Electrophoresis**—The molecular

---

**Table II**

Amino acid composition of IF-M1

Protein hydrolysates (22 and 72 hours) were analyzed on a Beckman model 120B amino acid analyzer as described under “Experimental Procedures.” Values for valine, isoleucine, and leucine were calculated by extrapolation to infinite hydrolysis time. Values for tryptophan, threonine, and serine were calculated by extrapolation to zero hydrolysis time. Cysteine was determined as S-sulfocysteine.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues per 65,000 daltons</th>
<th>Mol %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>61.4</td>
<td>10.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>12.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>20.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>34.3</td>
<td>9.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>61.4</td>
<td>10.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>36.9</td>
<td>6.3</td>
</tr>
<tr>
<td>Serine</td>
<td>42.7</td>
<td>7.3</td>
</tr>
<tr>
<td>Proline</td>
<td>40.4</td>
<td>6.9</td>
</tr>
<tr>
<td>Glycine</td>
<td>38.9</td>
<td>6.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>42.6</td>
<td>7.3</td>
</tr>
<tr>
<td>Cysteine</td>
<td>9.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>7.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Valine</td>
<td>44.4</td>
<td>7.6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>22.4</td>
<td>3.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>46.0</td>
<td>7.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>16.2</td>
<td>2.7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>19.4</td>
<td>3.3</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>9.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Total</td>
<td>585.7</td>
<td>100.0</td>
</tr>
</tbody>
</table>

---

**Fig. 5 (left).** Absorbance spectrum of IF-M1. Step 8 IF-M1 was dialyzed against 100 mM KCl, 10 mM Tris-HCl (pH 8.0), and 0.1 mM EDTA as described under “Experimental Procedure.” Dialysis buffer was used as the reference buffer. The spectrum was obtained using a Cary 16 recording spectrophotometer and quartz cuvettes with a path length of 10 mm.

**Fig. 6 (right).** Determination of the $s_{20,w}$ of purified (Step 8) IF-M1. Determination of $s_{20,w}$ using ultraviolet optics was performed as described under “Experimental Procedures.” Sedimentation was performed at 48,000 rpm at 22.5°. The data obtained were plotted as described by Chervenka (28). The observed $s$ value was 3.56. Correction for temperature and salts yielded an $s_{20,w}$ value of 3.35.

**Fig. 7.** IF-M1 molecular weight determination by sedimentation equilibrium centrifugation. Sedimentation equilibrium centrifugation at 9,000 rpm at 8° was performed as described under “Experimental Procedure.” The data were plotted as described by Chervenka (28). The $M(1 - \bar{v})$ value obtained was 17,870 which corresponds to a molecular weight of 66,400 using a $\bar{v}$ of 0.731 cm$^3$ per g.
FIG. 8. IF-M1 molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis of IF-M1 and standard protein markers was as described by Weber and Osborn (25), using 6% acrylamide gels (see "Experimental Procedure"). Protein bands were visualized using Coomassie brilliant blue stain followed by diffusion destaining in an acidic methanol solution.

Fig. 9. Molecular weight and Stokes radius determination of IF-M1 by Sephadex G-100 chromatography. A 400-μl sample, containing 1.25 mg each of cytochrome c, chymotrypsinogen A, bovine albumin, human γ-globulins, and 0.3 mg of Step 5 IF-M1, was chromatographed on a Sephadex G-150 column (0.9 × 60 cm) and 0.5-ml fractions were collected every 8 min. The elution position of the standard molecular weight markers was determined by absorbance at 280 nm. The elution position of IF-M1 was determined as activity (15-μl aliquots) in the poly(U) assay in the presence of saturating levels of complementary factors. A molecular weight for IF-M1 was determined by a comparison of the elution position of IF-M1 activity and the elution position of standard protein markers (A). Treatment of the data as described by Laurent and Killander (32) allowed the determination of the Stokes radius of IF-M1 (B).

FIG. 10. S value determination of IF-M1 by sucrose density gradient centrifugation. Samples (250 μl) containing 2.0 mg of ovalbumin, 1.2 mg of aldolase, 1.1 mg of catalase, 1.3 mg of β-galactosidase, and 0.2 mg of Step 5 IF-M1 were layered over linear 5 to 20% sucrose gradients and were centrifuged for 4.5 hours at 66,000 rpm at 4°C. The position of IF-M1 was determined by the activity of 20-μl aliquots in the poly(U) assay. The position of the standard protein markers was determined by absorbance at 280 nm using an ISCO density gradient fractionator and a Gilford model 2000 spectrometer with a flow cell attachment. S values for the standard protein markers were from published data (27).

Physical Properties of IF-M1 Activity

To ensure that the physical properties of homogeneous IF-M1 protein reflected the properties of IF-M1 in crude preparations, impure (Step 5) IF-M1 was used to obtain the s20,w and molecular weight of IF-M1 based on enzyme assay.

Sephadex G-150 Chromatography—Sephadex G-150 chromatography indicated IF-M1 had a molecular weight of 95,000 based upon the relative elution position of IF-M1 to standard protein markers (Fig. 9A). Treatment of the data as described by Siegel and Monty (26) indicated the Stokes radius of IF-M1 was 42 Å (Fig. 9B).

Sucrose Density Gradient Centrifugation—Based on a comparison of the sedimentation rate of IF-M1 activity to standard protein markers in linear sucrose density gradients, IF-M1 had a sedimentation coefficient of approximately 3.4 S (Fig. 10). With the independent determinations of s20,w and Stokes radius, it is possible to calculate the molecular weight of IF-M1. Assuming a δ of 0.2 g per g of protein (33), f/f0 is approximately 1.5; this indicates that IF-M1 is a highly asymmetric protein.

Biological Properties of IF-M1

Previous studies in this laboratory have shown that IF-M1 is required for the initiation factor-dependent "Mg2+ shift" in poly(U)-directed polyphenylalanine synthesis and the synthesis of globin chains. IF-M1 also is required in several model assay systems including endogenous mRNA-directed methionylpolypeptide synthesis.

Physical Properties of IF-M1 Activity

To ensure that the physical properties of homogeneous IF-M1 protein reflected the properties of IF-M1 in crude preparations, impure (Step 5) IF-M1 was used to obtain the s20,w and molecular weight of IF-M1 based on enzyme assay.

Sephadex G-150 Chromatography—Sephadex G-150 chromatography indicated IF-M1 had a molecular weight of 95,000 based upon the relative elution position of IF-M1 to standard protein markers (Fig. 9A). Treatment of the data as described by Siegel and Monty (26) indicated the Stokes radius of IF-M1 was 42 Å (Fig. 9B).

Sucrose Density Gradient Centrifugation—Based on a comparison of the sedimentation rate of IF-M1 activity to standard protein markers in linear sucrose density gradients, IF-M1 had a sedimentation coefficient of approximately 3.4 S (Fig. 10). With the independent determinations of s20,w and Stokes radius, it is possible to calculate the molecular weight of IF-M1. Assuming a δ of 0.2 g per g of protein (33), f/f0 is approximately 1.5; this indicates that IF-M1 is a highly asymmetric protein.

Biological Properties of IF-M1

Previous studies in this laboratory have shown that IF-M1 is required for the initiation factor-dependent "Mg2+ shift" in poly(U)-directed polyphenylalanine synthesis and the synthesis of globin chains. IF-M1 also is required in several model assay systems including endogenous mRNA-directed methionylpoly-
Fig. 11. AUG-directed fMet-tRNA binding catalyzed by purified IF-M1. Reaction mixtures containing 1.2 A260 units of salt-washed ribosomes, 0.3 A260 unit of AUG, 5 mM MgCl2, 10 pmol of [3H]fMet-tRNA, 100 mM KCl, 2 mM dithiothreitol, and 20 mM Tris-HCl (pH 7.5) were incubated for varying lengths of time at 23°C. Where indicated (●) 1.2 µg of purified (Step 8) IF-M1 were added. Reactions were stopped by dilution with wash buffer (100 mM KCl, 5 mM MgCl2, and 20 mM Tris-HCl, pH 7.5) and bound [3H]fMet-tRNA was collected on Millipore filters (type HA). In the presence of 1.2 µg of IF-1 with no added ribosomes, 0.11 pmol of [3H]fMet-tRNA was bound to filters.

Myosin synthesis. Finally, IF-M1 has been shown to be required for the AUG- and ribosome-dependent binding of fMet-tRNA and for the AUG-, ribosome-, and GTP-dependent binding of Met-tRNA (in the presence of IF-M2A and IF-M2B) (19). Inasmuch as the poly(U)-directed polyphenylalanine synthesis assay was used to locate the column elution position of IF-M1, it was not surprising that Step 8 IF-M1 was equally as active as Step 4, 5, 6, or 7 IF-M1 in this assay. To test the characteristics of the purified IF-M1, it was also assayed in AUG-directed fMet-tRNA binding to ribosomes. As can be seen in Fig. 11, 1.2 µg of IF-M1 bound 1.5 pmol of [3H]fMet-tRNA. Using 0.3 µg of IF-M1 it was possible to bind approximately 1.0 pmol of [3H]fMet-tRNA (data not shown). Under the conditions of limiting IF-M1, approximately 4 to 5 pmol of IF-M1 were required to bind 1 pmol of [3H]fMet-tRNA to ribosomes.

A comparison of various IF-M1 preparations revealed that whereas Step 8 (or 7) could bind fMet-tRNA, Step 5 (or 6) IF-M1 could bind approximately twice as much fMet-tRNA (Fig. 12). The binding of these four preparations was about equally dependent both on added AUG and ribosomes (data not shown), so that the binding observed could not be explained simply as nonspecific contaminants present in the less purified Step 5 and 6 IF-M1.

Identification of Contaminating fMet-tRNA Binding Factor—Inasmuch as the differences in fMet-tRNA binding became apparent following phosphocellulose chromatography, attempts were made to locate the apparent "extra" fMet-tRNA binding activity using phosphocellulose chromatography. An assay of column fractions (Fig. 1) revealed only one small peak of fMet-tRNA binding which was coincident with the IF-M1 activity detected by the poly(U) assay. However, when non-IF-M1 fractions were pooled (Fig. 1; tubes 1 to 22, 22 to 95, and 120 to 195) and concentrated, fMet-tRNA binding was found in the fraction from tubes 22 to 95. This binding activity was additive to that observed with saturating levels of Step 7 or 8 IF-M1 (Table III), thus simulating the fMet-tRNA binding observed with Step 5 or 6 IF-M1 (Fig. 12).

Heat Inactivation of IF-M1—Because of the different results obtained using the various IF-M1 preparations in fMet-tRNA binding and the isolation of an "extra" fMet-tRNA binding activity, additional studies were performed to test the properties of IF-M1 activity during different stages of purification.

![Graph showing fMet-tRNA binding to ribosomes](image-url)

**Fig. 12.** Comparison of crude and purified IF-M1 catalyzed [3H]fMet-tRNA binding. Reaction mixtures containing components listed in the legend to Fig. 11 were incubated for 2 min at 23°C with varying levels of either Step 5 or Step 8 IF-M1. Reactions were stopped by dilution with wash buffer and bound [3H]fMet-tRNA was collected on Millipore filters.

**Table III**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amount added (µg)</th>
<th>[3H]fMet-tRNA bound to ribosomes (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>0.41</td>
</tr>
<tr>
<td>IF-M1</td>
<td>1.9</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td>2.05</td>
</tr>
<tr>
<td></td>
<td>5.6</td>
<td>2.07</td>
</tr>
<tr>
<td>Binding factor</td>
<td>19.5</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>58.5</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>1.78</td>
</tr>
<tr>
<td></td>
<td>96.5</td>
<td>2.10</td>
</tr>
<tr>
<td>IF-M1 + binding factor</td>
<td>5.6/58.5</td>
<td>3.38</td>
</tr>
</tbody>
</table>

Downloaded from [http://www.jbc.org/](http://www.jbc.org/) by guest on October 23, 2017
Poly (U) Assay

MINUTES PRE-INCUBATED AT 45°C

TABLE IV

Inactivation of IF-M1 activity by N-ethylmaleimide

Samples were incubated in 10 mM N-ethylmaleimide for the times indicated. Following neutralization with 100 mM dithiothreitol, the various IF-M1 samples were assayed as described under "Experimental Procedure." The amount of IF-M1 used for the poly(U) assay was: Step 5, 12 µg; Step 6, 3.1 µg; Step 7, 0.20 µg; and Step 8, 0.15 µg. The amount of IF-M1 used for the fMet-tRNAf binding assay was: Step 5, 36 µg; Step 6, 9.3 µg; Step 7, 1.2 µg; and Step 8, 0.9 µg.

<table>
<thead>
<tr>
<th>IF-M1 Preparation</th>
<th>Time of incubation</th>
<th>Per cent inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>Poly(U) assay</td>
</tr>
<tr>
<td>Step 5</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>96</td>
</tr>
<tr>
<td>Step 6</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>98</td>
</tr>
<tr>
<td>Step 7</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>Step 8</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>100</td>
</tr>
</tbody>
</table>

Discussion

An eight-step procedure has been devised for the preparation of homogeneous IF-M1 from rabbit reticulocyte 0.5 M KCl ribosomal wash fraction (Table I). The overall purification was approximately 1500-fold with a yield of 15% of the original IF-M1 activity (using the poly(U) assay). The final IF-M1 preparation appeared homogeneous based on the following criteria: (a) single band using disc gel electrophoresis at pH 4.5 (Fig. 3); (b) single band using sodium dodecyl sulfate gel electrophoresis (Fig. 4); (c) uniformly sedimenting material in sedimentation velocity experiments; and (d) straight line plot from data obtained from sedimentation equilibrium experiments (Fig. 5). Molecular weight determinations based on both IF-M1 protein (equilibrium sedimentation, sodium dodecyl sulfate gel electrophoresis) and IF-M1 activity (sucrose density gradient sedimentation, gel filtration) indicated that IF-M1 is active as a single polypeptide chain with a molecular weight of approximately 65,000.

The purified IF-M1 had all of the biological properties previously cited in the poly(U) assay and in ribosome-dependent Phe-tRNAf and fMet-tRNAf binding (6, 16, 18, 19). However, the extent of fMet-tRNAf binding of purified IF-M1 was approximately 1/2 that of Step 5 or Step 6 IF-M1. Previous reports suggested that even higher levels of fMet-tRNAf binding could be observed in crude preparations; however, this binding was partially independent of AUG or ribosomes (16). The differences in fMet-tRNAf binding observed between Step 6 and Step 7 IF-M1 appeared to be due to a contaminating protein which elutes from phosphocellulose before IF-M1. Based upon enzyme assays and column chromatography, the non-IF-M1 fMet-tRNAf binding protein appears to have the following properties: (a) NEM insensitivity; (b) heat stability; (c) a molecular weight similar to reticulocyte IF-M1; (d) similar chromatographic characteristics to reticulocyte IF-M1 on DEAE- and CM-cellulose but not on phosphocellulose; and (e) an fMet-
tRNA\textsubscript{f} binding activity which is additive to that of IF-M1 and requires both AUC template and ribosomes.

The molecular weight of reticulocyte IF-M1 (65,000) is similar to that of E. coli IF-2 (80,000) (2, 33) and Artemia salina EIF-1 subunits (74,000; active EIF-1 has a molecular weight of 145,000) (9). The molecular weight of a rat liver cytosol factor (which binds Phe-, fMet-, and N-acetyl-Phe-tRNA\textsubscript{f} to 40 S subunits) is similar to that of reticulocyte IF-M1 based on a gel filtration molecular weight of 93,000 (8). Reticulocyte IF-M1 and E. coli IF-2 appear to have a rather high fractional ratio based on a comparison of molecular weight and elution position on Sephadex G-200, whereas A. salina EIF-1 appears to be a rather symmetrical molecule (9, 34).

In addition to similarities in molecular weight, most of the IF-M1-like binding factors have shown similar requirements in binding assays. For example, whereas E. coli IF-2 requires GTP for AUG-directed fMet-tRNA\textsubscript{f} binding, the eukaryotic IF-M1-like factors do not require GTP (6, 10, 35). Most, if not all, of the IF-M1-like factors studied to date are sensitive to N-ethylmaleimide inactivation (10, 35). It should be noted that crude reticulocyte IF-M1 preparations indicate the presence of an N-ethylmaleimide-insensitive fMet-tRNA\textsubscript{f} binding activity; however, this activity is separable from IF-M1 by phosphocellulose chromatography as described above.

Recent reports in the literature have suggested that the initiation of protein synthesis in both prokaryotes and eukaryotes may involve the formation of a soluble complex which contains initiation factor(s), GTP, and formylmethyl-tRNA\textsubscript{f} (11-15, 36-38). It has been reported previously by this laboratory that an IF-M1-dependent fMet-tRNA\textsubscript{f} complex is retained only on nitrocellulose filters in the presence of AUG and ribosomes and that an IF-M1, IF-M2A, and IF-312B-dependent Met-tRNA\textsubscript{f} complex is retained only on nitrocellulose filters in the presence of AUG, ribosomes, and GTP (10). However, recently we have separated an activity from IF-M3 which is required for methionyl-tRNA\textsubscript{f} synthesis and is capable of a G"IT-dependent binding of either Met-tRNA\textsubscript{f} or fMet-tRNA\textsubscript{f} to nitrocellulose filters in the absence of both AUG and ribosomes (15). This ribosome-independent binding factor (which we call IF-MP) appears to be similar to the one reported by Levin et al. (11), Dettman and Stanley (12), Gupta et al. (13), and Schreier and Stachelin (14). Inasmuch as the ribosome-independent binding factor is required for endogenous mRNA-directed methionyl-tRNA\textsubscript{f} synthesis and IF-M1 is required for ribosome-dependent binding of Met-tRNA\textsubscript{f}, it is not clear whether or not the ternary complex (binding factor-GTP-Met-tRNA\textsubscript{f}) is a necessary intermediate in protein biosynthesis.

Studies with natural mRNA-directed globin synthesis have yielded inconclusive results. Partially purified (Step 5) IF-M1 usually stimulates globin synthesis 2- to 3-fold, whereas more pure preparations appear less effective. On the other hand, initiation factor M1 does not appear to stimulate globin synthesis to any extent. The lack of stimulation by either factor seems due to the presence of both factors in the 100,000 x g lysate which is used as a source of aminocyl-tRNA synthetases. The precise role of these factors in natural mRNA-directed protein synthesis must await the further purification of the assay system.

Clearly, the elucidation of the step by step formation of an initiation complex will require the use of homogeneous factors. In particular, inconsistent results were obtained in fMet-tRNA\textsubscript{f} binding assays when comparing preparations of IF-M1 purified 80-fold (Step 6) with homogeneous IF-M1 (Step 8). The further purification of other rabbit reticulocyte initiation and elongation factors is being pursued currently.

Acknowledgments—The authors wish to thank Ms. Rebecca Rawis for excellent technical assistance; Mr. Bernard Ketauer for preparation of the rabbit reticulocyte lysate; Drs. Dante Piccinno, Philip Prichard, Hermann Graf, and Mr. Wayne Kemper for their assistance in the preparation of the complementary initiation and elongation factors used in this study; Drs. Richard Simpson, Darrell Liu, and Richard Shulman for help with the amino acid analysis; and Dr. Robert Simpson for assistance and discussions concerning the physical properties of homogeneous IF-M1.

REFERENCES

31. COHN, E. J., AND EDSELL, J. T. (1943) Protein, Amino Acids,


Purification and characterization of homogeneous protein synthesis initiation factor M1 from rabbit reticulocytes.
W C Merrick and W F Anderson


Access the most updated version of this article at http://www.jbc.org/content/250/4/1197

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/4/1197.full.html#ref-list-1