Purification and Physical Properties of Homogeneous Initiation Factor MP from Rabbit Reticulocytes*

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Initiation factor MP was purified 1570-fold with 67% recovery of total activity present in 0.5 M KC1 extracts of rabbit reticulocyte ribosomes. Initiation factor MP forms a ternary complex with Met-tRNAF and GTP or a binary complex with Met-tRNAF alone, the details of which are presented in the accompanying paper (Safer, B., Adams, S. L., Anderson, W. F., and Merrick, W. C. (1975) J. Biol. Chem. 250, 9076-9082). Initiation factor MP was homogeneous by the following criteria: (a) electrophoresis as a single band in gels of 5, 6, 7, 8, 9, and 10% acrylamide; (b) equilibration as a single band during isoelectric focusing; (c) sedimentation as a single symmetrical boundary during sedimentation velocity experiments; (d) linear plots of sedimentation equilibrium data; (e) symmetrical absorbance (at 280 nm) and activity profiles during DEAE-cellulose and Sephadex G-200 chromatography, and (f) symmetrical distribution of initiation factor MP during sucrose density gradient band sedimentation. The molecular weight of the initiation factor MP monomer (0.2 mg/ml) by low speed sedimentation equilibrium was 90,800.

Calculations based on the Stokes radius and sedimentation velocity show the existence of relatively stable 90,000-dalton monomers or 180,000-dalton dimers at low (0.1 mg/ml) and high (9.75 mg/ml) concentrations of initiation factor MP, respectively. Electrophoresis in sodium dodecyl sulfate gels indicates that initiation factor MP monomer is composed of two noncovalently linked subunits with molecular weights of 52,000 and 34,000. Despite a relatively normal amino acid composition and an isoelectric point of 6.4, initiation factor MP behaves as a basic protein, eluting from phosphocellulose at 650 mM KC1 (pH 7.9). Both ternary complex formation and methionyl-puromycin synthesis co-purify, indicating that a single protein is required for both activities.

Establishment of the initiation complex required for translation of natural messenger RNA in eucaryotic systems may involve either or both of two distinct proteins which bind Met-tRNAF. IF-M1* is required for GTP-independent Met-tRNAF binding to 40 S ribosome subunits in the presence of template (AUG or mRNA) and IF-M2B (1-3). IF-M1-like factors described from several different laboratories have been shown to bind fMet-tRNAF, Phe-tRNAF, or N-acetyl-Phe-tRNAF to ribosomes or 40 S subunits in the presence of the appropriate codon (AUG or UUU) (4-8). Alternatively a ternary complex composed of protein factor-GTP-Met-tRNAF has been described which appears to bind to the 40 S ribosomal subunit without the participation of other initiation factors or template (9-12).

Several laboratories have partially purified and characterized the protein factor (which we call IF-MP) which is capable of forming a ternary or binary complex with Met-tRNAF or GTP, or both (9-18). However, lack of homogeneity in these preparations does not allow exact comparison or identification of the specific protein factor(s) involved. In addition, partially purified fractions containing this factor(s) have been implicated in the reversal of hemin deficiency and double-stranded RNA inhibition of globin translation (19, 20), methionyl-puromycin formation (9, 18, 21-23), and specific binding to poly(A) and globin mRNA (17). IF-MP-like activity has also been isolated from ribonucleoprotein particles (17). To identify and characterize the protein factor(s) involved in these reactions, the 0.5 M KC1 ribosomal wash was fractionated for both methionyl-puromycin synthesis and ribosomal-independent Met-tRNAF binding activities. An 1570-fold purification of a single homogeneous protein factor (IF-MP) having the above two activities has been achieved. A rapid four-column procedure with a total recovery of 67% of the initial IF-MP activity and the physical characterization of IF-MP are presented. A detailed examination of the biological activities of IF-MP is reported in the accompanying paper.

EXPERIMENTAL PROCEDURES

Materials—Dithiothreitol, ATP, and GTP were purchased from Calbiochem. Rabbit liver [%5S]Met-tRNAF was prepared as previously described (24). The preparation was 8% pure with respect to methionine acceptance and 60 to 75% of the [%5S]methionine was precipitable by trichloroacetic acid. No tRNA,met was detectable by the procedure
of Bhaduri et al. (25). The specific activity of \( ^{38}S \)methionine obtained from New England Nuclear was 15,000 mCi/mmol. Dithiothreitol and GTP were obtained from Calbiochem. All other materials used were of reagent grade quality. DBAE-cellulose and phosphocellulose were obtained from Whatman (DE-23, DE-52, and P1) and were precomplexed according to the manufacturer’s recommendations. Proteins having known isoelectric point (pI) values used as standards in isoelectric focusing and proteins used as molecular weight standards for sodium dodecyl sulfate gel electrophoresis, standard gel electrophoresis, and gel filtration were: horse heart cytochrome c and rabbit muscle pyruvate kinase (Pharmacia Fine Chemicals, Inc.); bovine serum albumin, human \( \gamma \)-globulin, and bovine catalase (Schwarz/Mann); ovalbumin, \( \beta \)-galactosidase, horse-radish peroxidase, and DNAase I (Worthington Biochemical Corp.); horse heart myoglobin (Miles Laboratories, Inc.); and soy bean trypsin inhibitor, ceruloplasmin, phosphorylase \( a \) and \( b \), and bovine insulin, and lactoglobulin (Sigma).

### Assay of IF-MP Activity

**Assay of IF-MP Activity—**Assay of IF-MP activity was primarily measured by the GTP-dependent binding of \( ^{38}S \)/Met-tRNA, to nucleic cell filters. The standard 50-\( \mu \)l assay solution contained 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 10 \( \mu \)M GTP, and 0.5 \( \mu \)M Met-tRNA. The reaction was started by addition of IF-MP to the complete assay solution, which preincubation of the protein with GTP occurs in the absence of tRNA, irreversibly inactivates Met-tRNA, and the reaction mixture was incubated for 5 min at 0\( ^\circ \)C and the incubations were terminated by dilution with buffer and immediate vacuum filtration using nucleic cell filters (Millipore, type HA). The composition of the dilution buffer was 20 mM Tris (pH 7.5), 50 mM KCl, 10 mM MgCl\(_2\), and 1 mM methionine. The filters were then washed with three 2-ml aliquots of this buffer, and then dissolved and counted directly in 1 ml of scintillation fluid (Instabray, Yorktown Research). The counting efficiency was 80% for \( ^{38}S \) using a Packard Tri-Carb liquid scintillation spectrometer. MgCl\(_2\) and methionine were included in the wash buffer to lower the background of nonspecific binding of \( ^{38}S \)/Met-tRNA and free \( ^{38}S \)methionine to the nucleic filters. A detailed examination of Met-tRNA binding by IF MP will appear in the accompanying paper (26).

**Assay of IF-MP methionyl-puromycin dipeptide activity with endogenous mRNA was performed exactly as described previously (18).** Incubations in a total volume of 50 \( \mu \)l contained 5 \( \mu \)mol of \( ^{38}S \)/Met-tRNA, 0.5 \( \mu \)mol of puromycin, partially purified IF-M1, IF-M2A, and IF-M2B (3.4, 10, and 15 \( \mu \)g respectively), and 15 \( \mu \)g of protein. The final concentration of protein was determined from the absorption spectrum obtained with a Gilson model 240 spectrophotometer. Details of this purification procedure are presented under “Results.”

**Polyacrylamide Gel Electrophoresis—**Polyacrylamide gel electrophoresis of native IF-MP was performed in gels of indicated acrylamide concentration having a constant acrylamide/bisacrylamide ratio of 30/1 using the pH 8 Tris buffer system described in a publication from Canalc (27). The pH of the gel following pre-electrophoresis was 8.9 and protein samples were subjected to electrophoresis at 4\( ^\circ \)C at 1 mA/gel. Gels were stained with Coomassie brilliant blue according to the procedure of a Canalc publication as modified by dell (27). Binding activity was degraded by dilution with 5% acetic acid at 4\( ^\circ \)C. Bovine serum albumin, ovalbumin, soybean trypsin inhibitor, phosphorylase \( a \) and \( b \), catalase, and ceruloplasmin were used as standards for the analysis of the apparent IF-MP molecular weight and migration of the protein samples was confirmed by utilizing proteins with known pl values, such as myoglobin, ferritin, catalase, lactoglobulin, and bovine serum albumin (50), and by determining migration of the pH gradient and protein bands at several time points. Immediately following electrophoresis, gels were sliced and eluted with 0.5 \( \mu \)l of 50 mM KCl for 1 hour, and their pH was measured. Duplicate gels were stained immediately with fast green as described by the Medical Research Apparatus Corp. for rapid visualization of the polypeptide bands without prior removal of amphotiles.

### Sedimentation Velocity and Sedimentation Equilibrium

**Sedimentation Velocity and Sedimentation Equilibrium—**Ultracentrifugal studies of IF-MP were conducted in a Beckman model E analytical ultracentrifuge equipped with both schlieren and ultraviolet optics. Sedimentation velocity runs were conducted at 15-20\( ^\circ \)C and 12,000 rpm with all sedimentation coefficients corrected for the viscosity and density of water at 20\( ^\circ \). A Spinco An-H rotor and 12-mm, 2.5\( ^\circ \) double sector cells with Kel-F centerpieces were used.

Sedimentation equilibrium analyses were conducted at 10\( ^\circ \)C and 9,000 rpm using an An-D rotor. The final dialysis buffer was used both as the reference buffer and diluent for IF-MP. An initial 2-hour period of centrifugation at 12,000 rpm was employed to accelerate the approach to equilibrium. After attainment of equilibrium was determined by exact superimposition of the scans obtained at 280 nm at 36, 42, and 48 hours, the rotor was accelerated to 48,000 rpm to deplete the meniscus of protein. After 3 hours the rotor was decelerated to 9,000 rpm and the sectors were reelimed to a base-line at zero protein concentration. Analysis of the data was performed as described by Chervenka (31). The authors appreciate the assistance of Dr. Robert Simpson of the National Institute of Arthritis, Metabolism, and Digestive Diseases in performance and interpretation of the ultracentrifugal analysis.

### Determination of Stokes Radius and Apparent Molecular Weight by Gel Filtration

**Determination of Stokes Radius and Apparent Molecular Weight by Gel Filtration—**IF-MP was analyzed by ascending Sephadex G-200 chromatography on both preparative and analytical columns (2.6 \( \times \) 95 cm and 0.9 \( \times \) 80 cm, respectively). Both columns were eluted at a flow rate of 4 ml/cm/hour. Molecular weights of standard proteins were obtained from published data (32) and the Stokes radii derived by the equation \( a = kT/GnTD \) (\( a \), Stokes radius; \( k \), Boltzmann constant; \( T \), temperature; \( G \), viscosity; \( n \), density; \( D \), diffusion coefficient). Elution of bovine serum albumin was monitored by absorbance at 280 nm using a Zeiss spectrophotometer, with confirmation of IF-MP elution position by the Met-tRNA binding assay.

### Amino Acid Analysis

**Amino Acid Analysis—**Samples for amino acid analysis were made 20% in acetic acid and then chromatographed on a column (0.9 \( \times \) 55 cm) of F-30 (Bio-Rad) which had been equilibrated with 20% acetic acid. The tubes containing protein were pooled and then diluted 1:4 with distilled H\(_2\)O, 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.

Samples were hydrolyzed under vacuum in 4 \( \mu \)l methanesulfonic acid made 0.2% in tryptophan for either 22 or 72 hours as described by Liu and Inglis (34). Amino acid analysis was performed on a Beckman model 120B amino acid analyzer. All values were from duplicate determinations. 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 the complete assay solution. The authors thank Dr. Richard Simpson of the National Institute of Child Health and Human Development for kindly performing the amino acid analyses.

### Purification of IF-MP

**Purification of IF-MP

**Step 1: KCl (0.5 m) Ribosomal Wash—**Rabbit reticulocyte lysate and 0.5 \( \mu \)M KCl ribosomal wash were prepared as apparatus. The gel and buffer system described by the Medical Research Apparatus Corp. was used, with a final Ammonium concentration of 2% (equal parts of pH 5 to 8 and 6 to 8, LKB Ammonium solutions). The protein samples containing 10% glycerol and 2% pH 7 to 9 Ammonium were carefully layered under 100% of 2% pH 7 to 10 Ammonium to prevent denaturation by the 10 mM NaOH anolyte. The gels were run at 2 mA constant current/gel (3 mm (inside diameter) \( \times \) 75 mm (length)) until the applied voltage increased to 200 volts (approximately 30 min into the run) and then 200 volts was maintained.

Achievement of near equilibrium with respect to both the pH gradient and migration of the protein samples was confirmed by utilizing proteins with known pl values, such as myoglobin, ferritin, catalase, lactoglobulin, and bovine serum albumin (50), and by determining migration of the pH gradient and protein bands at several time points. Immediately following electrophoresis, gels were sliced and eluted with 0.5 \( \mu \)l of 50 mM KCl for 1 hour, and their pH was measured. Duplicate gels were stained immediately with fast green as described by the Medical Research Apparatus Corp. for rapid visualization of the polypeptide bands without prior removal of amphotiles.
Previously described (35, 36). This and all subsequent steps were performed at 2-4°C.

**Step 2: (NH₄)₂SO₄ Fractionation**—Seventeen grams of the 0.5 M KC1 ribosomal wash protein were concentrated by addition of neutralized (NH₄)₂SO₄ (50/1, (NH₄)₂SO₄/(NH₄)₂CO₃) to 70% saturation. The precipitated protein was collected by centrifugation, dissolved in 250 ml of Buffer A (20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, and 0.1 mM EDTA) containing 100 mM KC1 and dialyzed overnight against 50 volumes of this buffer. Initial studies showed that IF-MP is quantitatively precipitated between 40 and 50% saturation of (NH₄)₂SO₄. However, purification procedures established for other initiation factors present in the 0.5 M KC1 ribosomal wash require more extensive recovery of protein. Although the major purpose of this step is to concentrate the ribosomal wash protein, much of the contaminating hemoglobin is removed as well as unidentified inhibitors of the IF-MP-dependent Met-tRNAₐ₅ binding and methionyl-puromycin assays. This results in an apparent 300% increase of the total IF-MP activity present (Table I). For calculations of the purification achieved at each step, therefore, the specific activity of IF-MP is taken as unity following the (NH₄)₂SO₄ fractionation rather than the 0.5 M KC1 ribosomal wash.

**Step 3: DEAE-cellulose Chromatography**—Protein, 15,000 mg (15 mg/ml), was applied to a DEAE-cellulose column (Whatman DE23, 5 x 30 cm) equilibrated with Buffer A containing 100 mM KC1. The initiation factors IF-MP, IF-M₂A, IF-M₂B, and IF-M₃ and the elongation factors EF-1 and EF-2 are adsorbed while IF-M₁ is not. The protein was loaded and washed at 200 ml/hour until the absorbance at 280 nm was ≤ 0.1. Two subsequent batch elutions were then performed with Buffer A containing 210 and 400 mM KC1. The 100 to 210 mM KC1 batch elution containing IF-MP and IF-M₃ activity was used immediately in the next purification step. The two other fractions were collected and processed as previously described (37, 38).

**Step 4: Phosphocellulose Chromatography**—The 100 to 210 mM KC1 batch eluate was concentrated by precipitation with (NH₄)₂SO₄ to 80% saturation, dialyzed against 4 liters of Buffer B (50 mM Tris-HCl (pH 7.9), 1 mM dithiothreitol, and 0.1 mM EDTA) containing 100 mM KC1 and immediately applied to a phosphocellulose column (Whatman DE52, 1.6 x 5 cm) previously equilibrated with Buffer A containing 10% glycerol and 75 mM KC1. IF-MP activity was washed at 200 ml/hour until the absorbance at 280 nm was ≤ 0.05, at which point a linear salt gradient (500 x 500 ml) from 500 to 750 mM KC1 (in Buffer B) was started. A constant flow rate of 40 ml/hour was maintained and 5-ml fractions were collected. Both IF-MP-dependent Met-tRNAₐ₅ binding activity and methionyl-puromycin dipeptide synthesis reproducibly eluted at 650 mM KC1, coincident with a small absorbance peak at 280 nm. IF-MP is totally separated from IF-M₃ activity (39) which is not retained on phosphocellulose at 500 mM KC1, pH 7.9. As shown in Table I, a 30-fold purification is achieved by phosphocellulose chromatography.

**Step 5: Sephadex G-200 Chromatography**—Protein corresponding to IF MP activity from the phosphocellulose column was pooled and precipitated by dialysis against Buffer B containing neutralized (NH₄)₂SO₄ to 80% saturation. The precipitated protein was collected by centrifugation, dissolved in 5 ml of Buffer B containing 100 mM KC1 and 10% glycerol, and immediately loaded on a Sephadex G-200 column (2.6 x 95 cm) equilibrated with the same buffer. Ten per cent glycerol appears to stabilize CTP-dependent Met-tRNAₐ₅ binding activity and is present throughout the remainder of the purification procedure. A constant flow rate of 20 ml/hour was maintained with ascending chromatography and 5-ml fractions were collected. Three distinct absorbance peaks (at 280 nm) were observed (Fig. 1). Both Met-tRNAₐ₅ binding activity and methionyl-puromycin formation activity exactly coincided in the sodium dodecyl sulfate gel electrophoretogram of IF-MP following phosphocellulose chromatography.

**Step 6: DEAE-cellulose Chromatography**—In the final purification step, IF-MP activity from Sephadex G-200 was pooled, diluted to 75 mM KC1 with Buffer B containing 10% glycerol, and immediately applied to a DEAE-cellulose column (Whatman DE52, 1.6 x 5 cm) previously equilibrated with Buffer A containing 10% glycerol and 75 mM KC1. IF-MP activity was eluted with a linear salt gradient (150 x 150 ml) from 75 to 200 mM KC1 (in Buffer A plus 10% glycerol). Fractions (5 ml) were collected and processed as previously described (37, 38).

**TABLE I**

<table>
<thead>
<tr>
<th>Purification of rabbit reticulocyte IF-MP</th>
<th>Total Protein</th>
<th>Specific Activity*</th>
<th>Purification Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ribosomal wash, 0.5 M KC1</td>
<td>16,900 mg</td>
<td>0.11</td>
<td>1 fold</td>
</tr>
<tr>
<td>2. (NH₄)₂SO₄ Concentration, 0 to 80%</td>
<td>15,000 mg</td>
<td>0.35</td>
<td>1 fold</td>
</tr>
<tr>
<td>3. DEAE-cellulose (DE23)</td>
<td>825 mg</td>
<td>6.2</td>
<td>18 fold</td>
</tr>
<tr>
<td>4. Phosphocellulose (P11)</td>
<td>27.9 mg</td>
<td>187</td>
<td>530 fold</td>
</tr>
<tr>
<td>5. Sephadex G-200</td>
<td>9.6 mg</td>
<td>474</td>
<td>1300 fold</td>
</tr>
<tr>
<td>6. DEAE-cellulose (DE52)</td>
<td>6.45 mg</td>
<td>550</td>
<td>1570 fold</td>
</tr>
</tbody>
</table>

* One unit of activity is defined as the GTP-dependent binding of 1 pmol of Met-tRNAₐ₅ to nitrocellulose filters under standard IF-MP assay conditions (see "Experimental Procedures"). Ten picomoles of [³⁵S]Met-tRNAₐ₅ were present in each 50-μl reaction mixture.

**FIG. 1** Sephadex G-200 chromatography of IF-MP. Step 4 IF-MP (27.9 mg), concentrated by neutralized (NH₄)₂SO₄ was dissolved in 2 ml of Buffer B containing 100 mM KC1 and directly chromatographed on a Sephadex G-200 column (2.6 x 95 cm). IF-MP activity was assayed by both Met-tRNAₐ₅ binding and methionyl-puromycin formation, using 3- and 8-μl aliquots, respectively.
collected at a flow rate of 24 ml/hour. Both Met-tRNA$_\text{f}$ binding activity and methionyl-puromycin formation activity exactly coincided with a single symmetrical peak eluted at 125 mM KCl (Fig. 2). Protein corresponding to IF-MP activity was pooled and concentrated by dialysis against Buffer A containing (NH$_4$)$_2$SO$_4$ to 80% saturation. The precipitated protein was collected by centrifugation, dissolved in 1 ml of Buffer A containing 10% glycerol and 100 mM KCl, and dialyzed overnight against the same buffer.

Following clarification by centrifugation (10,000 × g for 20 min), aliquots of IF-MP were stored in liquid nitrogen at protein concentrations from 1 to 10 mg/ml. Both total Met-tRNA$_\text{f}$ binding (GTP-dependent plus GTP-independent) and methionyl-puromycin formation activities were stable for several months. However, as shown in the accompanying paper (26), the rate of formation of a stable IF-MP Met-tRNA$_\text{f}$ binary complex is increased. For this reason, all binding experiments were performed with freshly prepared IF-MP except where specifically noted. The overall purification of IF-MP achieved by this four-column procedure was 1,570-fold with a yield of 67% (Table I). The electrophoretic protein patterns in sodium dodecyl sulfate gels at these four stages of purification are presented in Fig. 3. The conditions under which the various initiation and elongation factors elute from DEAE-cellulose, phosphocellulose, hydroxylapatite, and Sephadex G-200 are summarized in Fig. 4 for comparison of our factor preparations with those of other laboratories.

Characterization of IF-MP

IF-MP prepared by this procedure was tested for homogeneity by the following procedures subsequently presented in detail: (a) standard analytical polyacrylamide gel electrophoresis; (b) isoelectric focusing polyacrylamide gel electrophoresis; (c) sodium dodecyl sulfate-polyacrylamide gel electrophoresis; (d) analytical ultracentrifugation; (e) sucrose gradient centrifugation; and (f) Sephadex G-200 chromatography.

Standard Polyacrylamide Gel Electrophoresis and Isoelectric Focusing Polyacrylamide Gel Electrophoresis—Polyacrylamide gel electrophoresis of native IF-MP in 5, 6, 7, 8, 9 and 10% acrylamide gels showed the presence of a single polypeptide band (Fig. 5). Analysis of the relative mobility of IF-MP in comparison to protein standards of known molecular weight by the method of Hendrick and Smith (28) indicated an apparent molecular weight of 150,000 (Fig. 5). Native IF-MP also migrates as a single band of protein in pH gradient polyacrylamide gels. A pI of 6.4 was obtained (Fig. 6).

Amino Acid Composition—The amino acid composition of homogeneous IF-MP is presented in Table II. Values for tryptophan, threonine, and serine were extrapolated to zero time based on data obtained at 24 and 72 hours of hydrolysis. One notable feature is a low molar per cent contribution by tyrosine and tryptophan. This accounts for the low extinction coefficient of P$_{14}$ determined to be 7.60. A $b$ of 0.734 cm$^2$/g was calculated from the amino acid composition by the method of Cohn and Edsall (41).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Homogeneous IF-MP analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 6.25% gels resolves two subunits with apparent molecular weights of 38,000 and 52,000 daltons. To test the validity of the standard proteins used to calibrate the semilog plot of molecular weight versus relative mobility, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with IF-MP and protein standards in gels at four different acrylamide concentrations. As shown in Fig. 7A, the relative mobilities of the phosphorylase, urease, lactic dehydrogenase, bovine serum albumin, ovalbumin, pyruvate kinase, and the larger subunit of IF-MP extrapolate to a common point at 0% acrylamide (%). This indicates an identical charge to mass ratio which is required for molecular weight analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In contrast the light subunit of IF-MP, and both the 25,000 and 50,000 molecular weight subunits of IgG extrapolate to lower %T values at 0%T. This would imply lower mass ratios and estimation of their molecular weight by this procedure is subject to error. A semilog plot of molecular weight versus the relative mobilities of these various standard proteins (Fig. 7B) indicates that the estimated molecular weights of the heavy and light IF-MP subunits are 52,000 and 32,000 to 38,000, respectively. Since both IgG subunits and the light IF-MP

Fig. 2. DEAE-cellulose chromatography of IF-MP. Step 5 IF-MP was diluted to 75 mM KCl and applied to a DEAE-cellulose column (1.6 × 5 cm) previously equilibrated with Buffer B containing 75 mM KCl and 10% glycerol. Following extensive washing of the column, IF-MP activity, as detected by both Met-tRNA$_\text{f}$ binding and methionyl-puromycin formation, was eluted with a 75 to 200 mM linear KCl gradient (300 ml).

Fig. 3. Sodium dodecyl sulfate electrophoresis of IF-MP at four stages of purification. The preparations of IF-MP shown are: A, step 3; B, step 4; C, step 5; and D, step 6. The amount of protein applied to each gel was 6, 4, 3, and 6 μg, respectively.
ELUTION FROM DEAE-CELLULOSE (DE-52)

100 200 300 mM KCl

ELUTION FROM PHOSPHOCELLULOSE (pH 7.9)

IF-M2B (a + b)

IF-M1

IF-M3

IF-M2A

IF-MP

EF-2

EF-1

IF-M1

IF-M2A

IF-MP

ELUTION FROM HYDROXYLAPATITE (HAPATITE CI)

IF-M2B (a + b)

IF-M3

IF-M2A

IF-MP

EF-2

IF-M1

IF-MP

ELUTION FROM SEPHADEX G-200

EF-1, IF-M3, IF-DF

EF-MP

IF-M2A

IF-M1, IF-2

IF-M2B (a + b)

Fig. 4. Representative elution positions of reticulocyte initiation and elongation factors from DEAE-cellulose, phosphocellulose, hydroxylapatite, and Sephadex G-200 presented for ease of comparison with other laboratory's purification procedures. For a detailed explanation of the nomenclature and biological functions of these factors, see Ref. 36, 40, and 45.

Fig. 5. Step 6 IF-MP was subjected to electrophoresis in 5, 6, 7, 8, 9, and 10% acrylamide gels and the relative mobility compared to that of protein standards of known molecular weight according to the procedure of Hendrick and Smith (28). Electrophoresis of 8 μg of native IF-MP as a single band in an 8% acrylamide gel and the apparent molecular weight of IF-MP obtained by this procedure (150,000) are shown.

subunit extrapolate to a common point at 0%T, use of the two IgG subunits as molecular weight standards for this subunit is validated and a value of 34,000 daltons is obtained. In agreement with a 1:1 stoichiometry of these subunits, the A₂₈₀ and A₂₈₀ absorption ratio of the two bands, in unstained and stained sodium dodecyl sulfate gels respectively, was 1.4 to 1.6. This would indicate a minimum molecular weight of 84,000 to 90,500 for an IF-MP monomer composed of one heavy and one light polypeptide chain.

Sedimentation Equilibrium Determination of Molecular Weight of IF-MP—The molecular weight of IF-MP was also determined by low speed sedimentation equilibrium centrifugation. Aliquots of IF-MP (0.2 and 0.3 mg/ml), dialyzed against Buffer B containing 100 mM KCl and 10% glycerol, were simultaneously centrifuged for 48 hours at 9,000 rpm at 10°C. Analysis of the sedimentation equilibrium data was performed according to the method of Chervenka (31). Two linear parallel lines were obtained (data not shown). A value of 24,160 was calculated for M₁-D₀ at both protein concentrations, yielding a molecular weight of 90,800.

Sedimentation Coefficient and Stokes Radius—During preparative Sephadex G-200 chromatography, an apparent molecular weight of 270,000 was obtained in contrast to a molecular weight of 90,800 obtained by sedimentation equilibrium. To test the possibility that this greater molecular weight resulted

FIG. 6. Isoelectric focusing of IF-MP. Eight micrograms of IF-MP were subjected to electrophoresis in 4% acrylamide gels containing 2% carrier ampholytes as described under “Experimental Procedures.” Analysis of the pH gradient was performed at 6 hours, at which time precipitation of IF-MP occurred.

TABLE II
Amino acid composition of IF-MP

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>IF-MP</th>
<th>IF-M1</th>
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</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>8.7</td>
<td>10.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Arginine</td>
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</tr>
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<td>Aspartic acid</td>
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</tr>
<tr>
<td>Total</td>
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</tbody>
</table>

FIG. 7. Sedimentation equilibrium analysis of IF-MP.
mentally a more exact sedimentation coefficient for the IF-MP dimer since dilution of IF-MP leads to dissociation to the monomeric state. At 9.75 mg/ml, IF-MP sedimented as a single symmetrical boundary as viewed with schlieren optics (Fig. 8A). At 0.2 mg/ml, homogeneity of IF-MP was also indicated by symmetry of the sedimenting boundary using ultraviolet optics (data not shown). At 5 mg/ml, however, a degree of asymmetry of the peak visualized with both schlieren and ultraviolet optics suggested the presence of at least two sedimenting species (Fig. 8B). An $s_{20,w}$ value of 5.7 was also obtained for IF-MP by sucrose gradient centrifugation (data not shown).

Homogeneous IF-MP was then chromatographed on an analytical Sephadex G-200 column (0.9 x 60 cm) equilibrated with Buffer A containing 100 mM KCl. The concentration of IF-MP applied was 1 mg/ml, compared to 15 to 20 mg/ml during preparative chromatography. An apparent molecular weight of 140,000 was obtained (Fig. 9). Treatment of the data obtained by both preparative and analytical Sephadex G-200 chromatography according to Siegel and Monty (42) indicated that the Stokes radius was 40 and 56 A for IF-MP chromatographed at a protein concentration of 1 and 15 mg/ml, respectively (Fig. 9B). From the relationship

$$M = \frac{a(6-p)N_{A}V_{p}}{(1 - \eta p)}$$

(where $a$ = Stokes radius, $\eta$ = viscosity, $N_{A}$ = Avogadro’s number, $s_{20,w}$ = corrected sedimentation coefficient, $V_{p}$ = partial specific volume, and $p$ = density of solution), the molecular weight of the IF-MP species obtained during Sephadex G-200 chromatography at high and low protein concentration can be calculated from these independent determinations of $s_{20,w}$ and Stokes radius (31, 42). At 9.75 mg/ml, the $s_{20,w}$ and Stokes radius of IF-MP were 6.65 and 56 A, respectively, yielding a molecular weight of 175,000. At 1 mg/ml, the corresponding values are 4.70 S and 45 A, indicating a molecular weight of 90,300. IF-MP can therefore exist as either a monomer or dimer, depending on protein concentration.

Fig. 7. Estimation of the molecular weights of the IF-MP subunits by sodium dodecyl sulfate gel electrophoresis. A, the relative mobilities of the two IF-MP subunits and standard proteins of known molecular weight extrapolated to 0% acrylamide (0% T). Acrylamide gels of 6.25, 8, 9.75, and 11.5% were utilized. The standard protein markers used were 1, phosphorylase b; 2, chymotrypsinogen; 3, bovine albumin; 4, IgG (heavy chain); 5, ovalbumin; 6, lactic dehydrogenase; 7, IgG (light chain). Points 5 and 7 correspond to the heavy and light subunits of IF-MP, respectively. B, estimation of the apparent molecular weight of IF-MP subunits as a function of acrylamide concentration. Treatment of the data was based on the procedure of Hendrick and Smith (28).

from (a) formation of relatively stable dimers or trimers of IF-MP at high protein concentrations or (b) a high degree of asymmetry in the IF-MP monomer resulting in a smaller elution volume from Sephadex G-200, two complementary approaches were utilized.

The $s_{20,w}$ of IF-MP was determined at 0.2, 1, 5, and 9.75 mg/ml. As seen in Fig. 8, $s_{20,w}$ decreases with decreasing protein concentration from 6.54 at 9.75 mg/ml to 4.70 at 0.2 mg/ml. This would support dimerization of IF-MP at high protein concentrations. It was not possible to obtain experimental a more exact sedimentation coefficient for the IF-MP dimer since dilution of IF-MP leads to dissociation to the monomeric state. At 9.75 mg/ml, IF-MP sedimented as a single symmetrical boundary as viewed with schlieren optics (Fig. 8A). At 0.2 mg/ml, homogeneity of IF-MP was also indicated by symmetry of the sedimenting boundary using ultraviolet optics (data not shown). At 5 mg/ml, however, a degree of asymmetry of the peak visualized with both schlieren and ultraviolet optics suggested the presence of at least two sedimenting species (Fig. 8B). An $s_{20,w}$ value of 5.7 was also obtained for IF-MP by sucrose gradient centrifugation (data not shown).

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Fig. 8. Determination of the $s_{20,w}$ as a function of IF-MP concentration. IF-MP at an initial concentration of 9.75 mg/ml was collected and serially diluted after each centrifugation run in a Beckman model E centrifuge to obtain additional data at 5, 1, and 0.2 mg/ml. Data were analyzed and corrected for temperature and salts as described by Chervenka (31). Samples were centrifuged at 60,000 rpm. Representative schlieren photographs of IF-MP are shown to illustrate sedimentation of A, the IF-MP dimer as a single symmetrical boundary (9.75 mg/ml); and B, the dimer-monomer equilibrium (5 mg/ml). Sedimentation is from left to right. Schlieren photographs were taken at 75 and 110 min, respectively.
A 67% recovery and 1570-fold purification of IF-MP was obtained by a four-column procedure. IF-MP was judged homogeneous by the following criteria: 
(a) migration as a single polypeptide band in polyacrylamide gels at 5, 6, 7, 8, 9, and 10% acrylamide (Fig. 5); 
(b) migration and equilibration as a single polypeptide band in pH gradient gels (isoelectric focusing) (Fig. 6); 
(c) movement in sedimentation velocity experiments as a single symmetrical boundary (Fig. 8); 
(d) linear plot of data obtained from the low speed sedimentation equilibrium run for the determination of molecular weight (data not shown); 
(e) symmetrical distribution of IF-MP in sucrose density gradient sedimentation (data not shown); and
(f) symmetrical elution profiles obtained by DEAE-cellulose and analytical Sephadex G-200 chromatography (data not shown). IF-MP distribution in criteria e and f were analyzed by Met-tRNA<sub>f</sub> binding activity, sodium dodecyl sulfate gel electrophoretograms, and absorbance at 280 nm.

Both gel filtration and sedimentation velocity experiments demonstrated that IF-MP can exist as either a 90,000-dalton monomer or 180,000-dalton dimer depending on the protein concentration. Enzyme activity appeared to be unaffected by either of these analyses (data not shown); therefore, it seems unlikely that the 90,000-dalton monomer represents an artificial breakdown product of the dimer. Studies are currently in progress to determine whether any physiologic significance can be attributed to this reversible association between monomer and dimer. Eucaryotic IF-MP isolated by this procedure appears to differ from the analogous procaryotic initiator fMet-tRNA<sub>f</sub> binding factor (IF-2) in that the monomeric form of IF-MP consists of two distinct protomeric subunits of 34,000 and 52,000 daltons. In contrast, the procaryotic IF-2 is active as a single polypeptide chain. In addition, IF-2-dependent fMet-tRNA<sub>f</sub> binding activity can be resolved into two distinct forms, IF-2-α and IF-2-β, corresponding to molecular weights of 98,000 and 83,000, respectively (43). The techniques we have used do not resolve two corresponding forms of IF-MP. Similar to other homogeneous eucaryotic initiation factors isolated in this laboratory (3, 44, 45) IF-MP is a highly asymmetrical molecule (Table III) having a relatively high content of basic and acidic amino acids (Table II).

Several laboratories have reported partial purification of protein(s) having GTP-dependent Met-tRNA<sub>f</sub> binding activity from the 0.5 M KCl ribosomal wash fraction of eucaryotic

### Table III

<table>
<thead>
<tr>
<th>Physical properties of IF-MP monomer</th>
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<tr>
<td><strong>Molecular weight:</strong></td>
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<tr>
<td>Sedimentation equilibrium</td>
</tr>
<tr>
<td>Stokes radius and s&lt;sub&gt;st,w&lt;/sub&gt;</td>
</tr>
<tr>
<td>SDS PAGE*</td>
</tr>
<tr>
<td><strong>Subunit composition</strong></td>
</tr>
<tr>
<td>1 x 52,000 protomer</td>
</tr>
<tr>
<td>1 x 34,000 protomer</td>
</tr>
<tr>
<td><strong>s&lt;sub&gt;st,w&lt;/sub&gt; (x 10&lt;sup&gt;−14&lt;/sup&gt;)</strong></td>
</tr>
<tr>
<td><strong>D&lt;sub&gt;st,w&lt;/sub&gt; (x 10&lt;sup&gt;−10&lt;/sup&gt;)</strong></td>
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<tr>
<td><strong>Stokes radius (Å)</strong></td>
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<tr>
<td><strong>f/f&lt;sub&gt;o&lt;/sub&gt;</strong></td>
</tr>
<tr>
<td><strong>pI</strong></td>
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<tr>
<td><strong>r&lt;sup&gt;2&lt;/sup&gt; (cm&lt;sup&gt;2&lt;/sup&gt;/g)</strong></td>
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* SDS PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
and the performance and interpretation of ultracentrifugal studies. For assistance in the performance and interpretation of ultracentrifugal studies and Drs. Richard Simpson, Darrell Liu, and Bryan Brewer for help with amino acid analyses. Appreciation is also extended to Mr. Wayne Kemper for assistance in the preparation of complementary initiation and elongation factors used in this study.

REFERENCES

27. Disc Electrophoresis (1972) Canaval, Rockville, Maryland
34. Liu, T., and Inglis, A. (1972) Methods Enzymol. 25, 55-60
39. Frichard, P. M., Picciano, D. J., Laycock, D. G., and Anderson,
Purification and physical properties of homogeneous initiation factor MP from rabbit reticulocytes.
B Safer, W F Anderson and W C Merrick


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