A New Method for the Purification of Initiation Factor F2 in High Yield, and an Estimation of Stoichiometry in the Binding Reaction*

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SUMMARY

A simple method is described for purifying protein synthesis initiation factor F2 which involves ammonium sulfate fractional precipitation of a 0.5 M NH₄Cl wash of the ribosomes, followed by chromatography on cellulose phosphate and then on Sephadex G-200. Cellulose phosphate chromatography is an especially effective method, producing a 36-fold purification. The over-all yield of F2 is 23%, the over-all purification is 115-fold, and the final product shows one band on analytical disc gel electrophoresis. With this preparation it was shown that 1 molecule of F2 could stimulate the binding of up to 7 N-formylmethionyl tRNA molecules to ribosomes. This indicates that F2 is released from the ribosome after catalyzing the binding reaction and can then be reused.

The factors involved in the initiation of protein synthesis were first described in 1966 by Stanley, Salas, Wahba, and Ochoa (1), by Revel and Gros (2), and by Eisenstadt and Brawerman (3). When ribosomes were washed with high salt buffers (1), the factors separated, leaving ribosomes which were inactive for protein synthesis coded by natural messenger RNA. The initial discoveries were followed by the fractionation of the original material and the description of the three initiation factors F1 (A), F2 (C), and F3 (B) (4-7). The reactions which are catalyzed by these three factors are now known to involve the binding of the mRNA and fMet-tRNA to the 30 S ribosome (5, 6, 8-11) and the hydrolysis of GTP (12-15), but their exact sequence and nature are not yet fully understood. Attempts to elucidate these individual reactions involved in peptide chain initiation pure Factor F2 is needed. F2 of a low degree of purity has generally been prepared by high salt washes (0.5, 1.0, or 2.0 M NH₄Cl) of ribosomes and separated from the other initiation factors by chromatography on DEAE-cellulose (4-7). An earlier procedure developed in this laboratory (20, 27), using DEAE-cellulose chromatography, elution from pulverized glass columns, and sucrose gradient centrifugation yielded 30 to 70% pure F2 preparations which were, however, very unstable. A description of a new method for preparing a more stable F2 which shows one band on analytical disc gel electrophoresis is presented here. Since this work was begun, two quite different procedures for purifying F2 have been published (25, 28) and a discussion of them is included.

MATERIALS AND METHODS

Bacterial cell extracts (S-30) were prepared from frozen Escherichia coli K12 essentially by the method of Capecci and Gussin (29) except that 1.5 weight equivalents of alumina were used and the extraction buffer consisted of 20 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, and 1 mM dithiothreitol. Crude ribosomes were obtained from the S 30 by centrifuging at 60,000 rpm for 3 hours at 4° in a Spinco rotor No. 65 or No. 60 Ti. For use in the F2 assay, crude ribosomes were washed twice with 50 mM Tris-HCl, pH 7.4, 500 mM NH₄Cl, 20 mM magnesium acetate, and 1 mM dithiothreitol, and finally with 10 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, 30 mM NH₄Cl, and 1 mM dithiothreitol. The ribosomes were collected after each washing by centrifuging for 3 hours at 60,000 rpm and were stored at a concentration of 80 mg per ml in the latter buffer at 0°. Ribosomes prepared in this way are about 50% active for fMet-tRNA binding in the presence of excess F2. Unfraction-
ated labeled fMet-tRNA (formyl-\(^{3}H\)-methionyl-tRNA) was prepared essentially as previously described (13) except that the prior purification of the uncharged tRNA was omitted. ApUpG synthesis has been described by Sundararajan and Thach (30). Factor F1, a gift from Dr. J. Hershey, was purified by the method of Hershey, Dewey, and Thach (23) as far as the cellulose phosphate chromatography step. GTP (A grade) was a product of Calbiochem.

Log phase frozen E. coli K12 was obtained from Grain Processing Company, Muscatine, Iowa (\(1\) log phase) and from General Biochemicals. \(^{3}H\)-Met-hionine was obtained from New England Nuclear. DNase (RNase free) was a product of Worthington. Alumina was a product of Norton Abrasives, Worcester, Massachusetts. Dithiothreitol (Cleland's reagent) was obtained from Calbiochem. Acrylamide (electrophoresis grade) and \(N, N', N''\)-tetramethylethylenediamine in a 0.45 M potassium acetate buffer were obtained from Acros. Alumina was a product of Norton Abrasives, Worcester, Massachusetts. Diethylenetriamine was a product of Mann. Acrylamide (electrophoresis grade) and \(N, N', N''\)-methylenebisacrylamide from a mixture containing 1 part of 30% acrylamide, 0.8% \(N, N', N''\)-tetramethylethylenediamine were obtained from Eastman.

Quantitative Assay for Factor F2—The quantitative assay for F2 is based on its ability to stimulate the binding of fMet-tRNA to ribosomes in the presence of ApUpG and GTP. The standard assay contained in a volume of 50 ml of 50 mM Tris-HCl, pH 7.4, 100 mM \(\mathrm{NH}_4\)Cl, 5 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM ApUpG, 1 mM GTP, 0.8 \(\mu\)g of F1, 30 \(\mu\)moles of unfraccion-ated washed ribosomes, 30 to 40 \(\mu\)moles of \(^{3}H\)-fMet-tRNA with 5000 Ci per mole, and 2 to 20 \(\mu\)l of the F2 preparation being tested. Incubation was carried out for 15 min, at 25° and the reaction was stopped by the addition of 1 ml of ice-cold buffer containing 90 mM Tris-HCl, pH 7.4, 100 mM \(\mathrm{NH}_4\)Cl, and 10 mM magnesium acetate. The solution was then filtered through a Millipore filter (31); the filter was washed with the last mentioned buffer and dried. The radioactivity retained by the filter was measured in a Beckman LS 250 scintillation counter using a toluene scintillation fluid.

Under these conditions the amount of fMet-tRNA bound to the ribosomes was proportional to the concentration of F2 within a limited range (Fig. 1). The upper limit of the linear range was found to vary with the batch of ribosomes used in the assay and was therefore determined for each new preparation of ribosomes. Fig. 1, for example, shows an assay system linear up to 6 \(\mu\)moles of fMet-tRNA bound. All assays for any one purification experiment were carried out with the same preparation of ribosomes.

A unit of F2 activity is defined as 1.0 \(\mu\)mole of fMet-tRNA bound to ribosomes in the standard assay. Specific activity which was used to monitor the effect of purification methods is defined as the number of units per 1.0 \(\mu\)g of protein.

Protein was determined either by measuring the optical density at 280 and 260 nm and calculating according to Kalckar (32) or by Folin determination according to Lowry (33) using bovine serum albumin as the reference protein.

Polyacrylamide Disc Gel Electrophoresis—An analytical temperature-regulated disc electrophoresis apparatus (Buchler Polyanalysit) was used to analyze the F2 preparations following the different purification steps. Electrophoresis was carried out in an acidic buffer system (pH 4.3) using a modification of the procedure of Reisfeld, Lewis, and Williams (34). Because F2 has been reported to contain free sulfhydryl groups (28) gels were, prepared free of ammonium persulfate to avoid a possible source of artifacts (35, 36). Riboflavin and light (two Buchler fluorescence lamps) alone (30) were used to initiate polymerization of the gels. Gels were prepared and subjected to electrophoresis in glass tubes (0.8 × 7.5 cm). The lower gels were polymerized from a mixture containing 1 part of 30% acrylamide, 0.8% \(N, N', N''\)-methylenebisacrylamide, and 1 part of 0.24% \(N, N', N''\)-tetramethylethylenediamine in 1.5 mM potassium acetate buffer, pH 4.3, and 2 parts of 0.2% riboflavin. The upper gels were polymerized from a mixture containing 1 part of 10% acrylamide, 0.8% \(N, N', N''\)-methylenebisacrylamide, and 1 part of 0.3% \(N, N', N''\)-tetramethylethylenediamine in 0.45 mM potassium acetate buffer, pH 6.7, and 2 parts of 0.2% riboflavin. The anodal buffer with a pH of 4.5 contained 0.12 mM acetic acid and 0.3 mM \(\beta\)-alanine. The cathodal buffer with a pH of 4.3 contained 0.05 M potassium acetate buffer. After polymerization 50 or 100 \(\mu\)l of the concentrated protein samples were layered onto the gels. Gels were subjected to electrophoresis at 3 ma per gel for 4.5 hours. Following the electrophoresis the gels were fixed in 10% trichloracetic acid, stained in 0.05% Coomassie blue in 5% trichloroacetic acid overnight, and then destained in 5% trichloroacetic acid.

RESULTS AND DISCUSSION

Purification of Factor F2

Step 1. Wash (0.5 × \(\mathrm{NH}_4\)Cl) of Ribosomes—The crude ribosomes obtained from 80 g of frozen E. coli K12 were suspended in 60 ml of buffer containing 60 mM Tris-HCl, pH 7.4, 500 mM \(\mathrm{NH}_4\)Cl, 20 mM magnesium acetate, and 1 mM dithiothreitol and...
allowed to stand overnight. The washed ribosomes were then removed from the solution containing F2 by centrifuging at 60,000 rpm for 3 hours. In five independent purification experiments the supernatant (designated Step 1 F2) contained an average of 325 mg of protein in a volume of 55 ml. The average specific activity was 0.41 unit per µg. The 0.5 M NH₄Cl wash of the ribosomes was prepared for the fractionation of Step 1 F2 instead of the 1.0 M NH₄Cl wash previously used in this and other laboratories because this concentration is sufficient to separate F2 from the ribosomes; it also removed fewer contaminating proteins. The resulting ribosomes are free of F2 activity and the yield of Step 1 F2 was the same with the 0.5 M NH₄Cl wash as with the 1.0 M wash. Washing with 0.5 M NH₄Cl has the advantage of yielding a Step 1 preparation with a higher specific activity. The average total of 135,000 units of F2 obtained in this step is considered 100% for the calculation of the yield in further steps (see Table I).

Step 2. Fractional Precipitation with (NH₄)₂SO₄—Step 1 F2 consisting of an average of 324 mg of protein in 55 ml was subjected to fractional precipitation. A solution (29.5 ml) saturated at 4°C with (NH₄)₂SO₄ and containing 50 mM Tris-HCl, pH 7.4, and 1 mM dithiothreitol was added slowly with gentle stirring to the F2 solution. (Final concentration, 35% saturation.) After standing for 40 min the precipitate was collected by centrifuging at 15,000 rpm for 10 min. The supernatant (81 ml) 24 ml of the saturated (NH₄)₂SO₄ solution were added giving a final concentration of 50% saturation. The resulting precipitate was collected by centrifuging. The second fraction collected (35 to 50% saturation) contained an average (from five different experiments) of 32% of the Step 1 F2 activity and 108 mg of protein. This represents a 1.5-fold purification in terms of protein. In addition most of the material absorbing at 260 nm does not precipitate at 50% saturation and is therefore separated from the F2 in this step. (Thus the ratio of A₂₆₀/A₅₄₀ increased from 0.64 to 1.10.) In all five experiments low levels of F2 activity were discarded with both the first fraction precipitating at 0 to 35% (NH₄)₂SO₄ and a third fraction precipitating at 50 to 70%. The average F2 content for the former is 5.3% of the Step 1 F2 and for the latter 5.6%. The Step 2 F2 preparation contained an average total of 70,000 units of F2 with an average specific activity of 0.64 unit per µg of protein.

Step 3. Chromatography on Cellulose Phosphate—In a typical experiment 108 mg of protein from Step 2 which contained 52,600 units of F2 in 3.8 ml was dialyzed against Buffer A (50 mM Tris-HCl, pH 7.4, 20 mM NH₄Cl, and 1 mM dithiothreitol) and applied to a cellulose phosphate column (1.2 x 13 cm). A linear gradient of 90 ml of Buffer A and 90 ml of Buffer B (50 mM Tris-HCl, pH 7.4, 500 mM NH₄Cl, and 1 mM dithiothreitol) was applied and 110 fractions were collected at the rate of 0.3 ml per min (Fig. 2). The breakthrough of the column, containing most of the protein, contained less than 1% of the F2 activity which had been applied to the column. The F2 activity peak was found in Fractions 49 to 60. These 12 active fractions which were later combined contained 34,400 units or 66% of the F2 activity which had been applied to the column. (The average recovery of F2 activity in six independent similar cellulose phosphate purification experiments was 80%) The combined Step 3 F2 in this experiment contained only 2.0 mg of protein with a specific activity of 17 units of F2 per µg protein. Cellulose phosphate chromatography is therefore the most effective step in this purification procedure.

**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total units</th>
<th>Yield</th>
<th>Protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 0.5 M NH₄Cl wash of ribosomes</td>
<td>64.0</td>
<td>134,500</td>
<td>100</td>
<td>325</td>
<td>0.41</td>
</tr>
<tr>
<td>2. (NH₄)₂SO₄ fractionation</td>
<td>3.8</td>
<td>70,200</td>
<td>52</td>
<td>109</td>
<td>0.64</td>
</tr>
<tr>
<td>3. Cellulose phosphate chromatography</td>
<td>22.0</td>
<td>34,400</td>
<td>24</td>
<td>2.0</td>
<td>17.0</td>
</tr>
<tr>
<td>3a. Concentration using ultrafiltration</td>
<td>4.5</td>
<td>34,000</td>
<td>24</td>
<td>2.1</td>
<td>16.0</td>
</tr>
<tr>
<td>4. Gel filtration on Sephadex G-200</td>
<td>44.0</td>
<td>33,000</td>
<td>23</td>
<td></td>
<td>47.0</td>
</tr>
<tr>
<td>4a. Concentration of a small portion using ultrafiltration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>47.0</td>
</tr>
</tbody>
</table>

* Average results of five experiments are reported.

+ Values obtained in a single experiment.

The purification achieved in the experiment described here is 36-fold. As shown in Fig. 4, analytical disc gel electrophoresis of Step 3 F2 showed the presence of one major and two minor bands.

The volume of the Step 3 F2 was reduced from 22 to 4.5 ml through ultracentrifugation. The filtration was carried out at 0°C through dialysis tubing into a hematized 2-liter suction flask. Ninety-five per cent of the F2 activity was recovered.

**Step 4. Gel Filtration on Sephadex G-200**—Because preliminary experiments had shown that a purification of 5- to 10-fold could be obtained using Sephadex G-200 filtration when the starting material was F2 of a low degree of purity (Fig. 3A), this method was used as the final step in the purification of F2.

In a typical experiment 2 mg of combined concentrated F2
from Step 3 with a specific activity of 17 units per µg was applied to a Sephadex G-200 column (2.7 × 100 cm) equilibrated with 50 mM Tris-HCl, pH 7.4, 300 mM NH₄Cl, and 1 mM dithiothreitol. The column was eluted with the same buffer at the rate of 1 ml per 5 min and 4.0-ml fractions were collected. F2 activity was recovered in a single peak which followed the void volume (Fig. 3B). Comparison of the elution profiles in Fig. 3 indicates that the final pure product, Step 4 F2, is probably identical in size and shape with the bulk of F2 present in crude extracts. The active fractions (30 to 40) which were combined contained most of the F2 activity applied to the column. (The average yield on G-200 purification of six F2 preparations of varying starting composition was 95%.) Because the Step 4 F2 was extremely dilute, neither protein concentration nor specific activity could be determined accurately. The activity recovered was 33,000 units which represents an over-all yield for the four-step purification procedure of 23% (Table I). For most uses no further concentration was necessary.

However, in order to determine specific activity and to do gel electrophoresis a portion of Step 4 F2 was concentrated 5-fold using ultrafiltration. The recovery of F2 activity using this technique averages about 66% for Step 4a; the reason for this loss is not known. However, recent experiments have shown that a higher recovery is obtained when a phosphocellulose column is used for concentration (23).

The specific activity found for the concentrated material (designated Step 4a) is 47 units per µg protein. Because of the loss of F2 activity on concentration, one can conclude that the specific activity of Step 4 F2 is at least 47 and possibly slightly higher.

On analytical disc electrophoresis at pH 4.3 Step 4a F2 was found to show a single band corresponding to the major component of Step 3a material (Fig. 4). This indicates that the Step 4 F2 is more than 90% pure.

**Binding of fMet-tRNA to Ribosomes**

As has been reported for other F2 preparations, the binding of fMet-tRNA to ribosomes catalyzed by Step 4a F2 is dependent on the presence of the messenger and on GTP (Table II), but is only partially dependent on Factor F1 (Table III).

**Comparison with Other Methods**

Since this work was begun, two other procedures have been published for preparing F2 (25, 28). Both of these procedures

**Table II**

<table>
<thead>
<tr>
<th>Standard assay using 0.09 µg of Step 4a F2.</th>
</tr>
</thead>
<tbody>
<tr>
<td>fMet-tRNA bound</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Complete assay</td>
</tr>
<tr>
<td>Minus AUG</td>
</tr>
<tr>
<td>Minus GTP</td>
</tr>
</tbody>
</table>

**Table III**

<table>
<thead>
<tr>
<th>Standard assay with 0.11 µg of Step 4a F2 and 0.8 µg of F1. The blank obtained in the absence of both factors (0.48) has been subtracted.</th>
</tr>
</thead>
<tbody>
<tr>
<td>fMet-tRNA bound</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Plus F2 plus F1</td>
</tr>
<tr>
<td>Plus F2 minus F1</td>
</tr>
<tr>
<td>Minus F2 plus F1</td>
</tr>
</tbody>
</table>
TABLE IV

Dependence of fMet-tRNA binding to ribosomes on method of preparing ribosomes

<table>
<thead>
<tr>
<th>fMet-tRNA bound</th>
<th>Ribosomes washed with 0.5 M NH₄Cl</th>
<th>Ribosomes washed with 1.0 M NH₄Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.07 μg of F2</td>
<td>0.11 μg of F2</td>
</tr>
<tr>
<td>μmoles</td>
<td>3.27</td>
<td>2.17</td>
</tr>
<tr>
<td></td>
<td>5.51</td>
<td>3.31</td>
</tr>
</tbody>
</table>

yield F2 which shows a single band on disc gel electrophoresis. The procedure of Chae, Mazumder, and Ochoa (28) involves six steps and gives a 1% yield of F2 which shows an AUG-dependent binding of 16 μmoles of fMet-tRNA to ribosomes per μg of F2 in 15 min at 25°. For the procedure of Heraberg, Lelong, and Revel (25) which requires five steps and for which recovery of 2.2% can be calculated, an AUG-dependent binding of 3.3 μmoles of fMet-tRNA per μg of F2 in 10 min at 25° was found. Our four-step purification procedure gives a 23% recovery of F2 which shows an AUG-dependent binding of 47 μmoles of fMet-tRNA to ribosomes per μg of F2 in 15 min at 25°.

A partial explanation of the different final specific activities might lie in the small differences in concentration of the components of the assay system. Another possible cause of this variation is the presence of inactivated F2 which is not distinguishable on analytical disc gel electrophoresis. Finally, the possibility that the method of ribosome preparation might influence the value of specific activity was considered. To test this possibility the standard assay was carried out with standard ribosomes and those which had been washed twice with 1.0 M NH₄Cl (plus 50 mM Tris-Cl, pH 7.4, 40 mM magnesium acetate, and 1 mM dithiothreitol) (Table IV). Table IV shows that the extent of fMet-tRNA binding to ribosomes stimulated by a given amount of F2 varies with the method used to prepare the ribosomes. Thus, we conclude that the specific activities determined for F2 vary with the preparation of ribosomes used in the assay and that this fact, in addition to the other possibilities mentioned above, may be responsible for the different values reported by different groups.

Recycling of F2

As shown in Table I, the purest F2 has a specific activity of 47 in the standard assay. Using the known molecular weight of F2 of approximately 70,000 (20, 28) one can calculate that under the standard assay conditions 0.07 μg or 1.0 μmole of F2 stimulates the binding to ribosomes of 3.3 μmoles of fMet-tRNA. To see if the ratio of fMet-tRNA bound to F2 could be further increased experiments were carried out in which a slightly modified assay mixture, containing higher concentrations of ribosomes and fMet-tRNA (see legend to Fig. 5), was incubated at 25° for 30 min. Under these nonstandard assay conditions 1.0 μmole of F2 was found to stimulate the binding of 7.5 μmoles of fMet-tRNA to ribosomes. Furthermore, an examination of the time course (Fig. 5) showed that the binding of fMet-tRNA was still increasing at 30 min. From the above data it is concluded that F2 may be recycled over seven times in the fMet-tRNA binding assay. The finding of F2 recycling means that F2 is released from the ribosome either simultaneously with or after the binding of fMet-tRNA and before the first peptide bond is formed—indeed before the second aminoacyl-tRNA is bound to the ribosome. Similar results have recently been reported by Chae, Mazumder, and Ochoa (18). Thus, it is clear that F2 is similar to F1 in that it is released from the ribosome after serving its function (23, 27).

Conclusion

A four-step method is described for preparing F2 in 23% yield which shows one band on analytical disc gel electrophoresis. From the finding that 1.0 μmole of the purest F2 catalyzes the AUG-dependent binding to ribosomes of 7.5 μmoles of fMet-tRNA the conclusion is drawn that F2 is released from the ribosome and recycled under the conditions of protein synthesis initiation.

REFERENCES

A New Method for the Purification of Initiation Factor F2 in High Yield, and an Estimation of Stoichiometry in the Binding Reaction
Eileen Remold-O'Donnell and Robert E. Thach