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Isolation and Properties of Polypeptide Chain Initiation Factor FII from *Escherichia coli*: Evidence for a Dual Function

JERRY S. DUBNOFF* AND UMADAS MAITRA†

Department of Developmental Biology and Cancer, Division of Biology,
Albert Einstein College of Medicine, Yeshiva University, Bronx, New York 10461

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ABSTRACT **Initiation factor FII (F_3, factor B) has been purified from *Escherichia coli* Q13 to apparent homogeneity. It is a heat-stable basic protein, consisting of a single polypeptide chain of molecular weight 21,000. FII is required, in addition to the other factors FI and FIII, for the formation of a 70S complex containing fMet-tRNA, poly(U,G) (used as a model messenger RNA), and the ribosome. FII also is capable of dissociating 70S ribosomes to 30S and 50S subunits. Evidence is presented that both activities are mediated by the same protein.**

The initiation of protein synthesis in *Escherichia coli* proceeds with the binding of mRNA and fMet-tRNA to the 30S ribosomal subunit, with subsequent addition of the 50S subunit to form the 70S initiation complex (1). The overall reaction requires several protein factors that are loosely associated with ribosomes, from which they can be dissociated by high salt treatment. We have previously reported the separation of three such initiation factors, which we have designated FI, FII, and FIII in order of their elution from DEAE-cellulose (2–4). They appear to correspond to the factors F_1, F_3, and F_2 isolated in Ochoa's laboratory (5) and to factors A, B, and C described by Revel and coworkers (6).

Reports from other laboratories have assigned various roles to the factor corresponding to FII (F_3, factor B) including (*a*) specific recognition of natural, as opposed to synthetic mRNA (5–7) and (*b*) binding of mRNA to ribosomes in the absence of the factor corresponding to FIII (F_2, factor C) (5, 8). Several groups have also described a 'dissociation factor' (DF) in *E. coli* extracts which promotes dissociation of 70S ribosomes into subunits (9–13). The ribosome dissociation activity was reported to cofractionate with the initiation factors but the question of possible identity of DF with a specific initiation factor has not been completely resolved. Subramanian *et al.* (10) found that DF eluted from DEAE-cellulose in the region corresponding to FII (F_3, factor B). A more highly purified preparation of this factor also possessed DF activity, whereas factor preparations corresponding to FI and FIII did not. The possibility that separate proteins in the FII preparation might be responsible for the two activities could not be excluded. Miall *et al.* (12), after purifying a ribosome dissociation activity to apparent homogeneity, speculated that DF might correspond to FI rather than to FII.

In the studies presented below, the random copolymer poly(U,G) has been used as a model messenger RNA for the study of the formation of the 70S initiation complex and for the purification of FII, yielding a preparation that is electrophoretically homogeneous. With poly(U,G) as messenger, FII is required in addition to factors FI and FIII for maximum incorporation of fMet-tRNA into a 70S complex; thus FII is not specific for "natural mRNA." Evidence is also presented that all three factors and fMet-tRNA are required for the maximum incorporation of messenger [^{3}H]poly(U,G) into a 70S complex, and that the requirement for FIII (F_2, factor C) is almost absolute. FII also exhibits ribosome dissociation activity, while the other factors are virtually inactive at comparable concentrations. Additional evidence is presented that the same protein promotes both fMet-tRNA binding to ribosomes and dissociation of 70S ribosomes into subunits.

Abbreviations: DF, dissociation factor; SDS, sodium dodecyl sulfate; GMP-PCP, guanylyl(β,γ-methylene)diphosphonate.

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† Established Investigator of the American Heart Association.

MATERIALS AND METHODS

Poly(U,G) (U:G = 3:1), unfractionated tRNA (*E. coli* B), and GTP were obtained from Schwarz BioResearch. fMet-tRNA was prepared by charging tRNA with methionine (^{3}H-labeled, 2500 cpm/pmol from New England Nuclear Corp. or unlabeled, from Schwarz BioResearch) in the presence of N^{10}-formyltetrahydrofolate, by means of a crude *E. coli* supernatant protein fraction. [^{3}H]poly(U,G) was prepared with purified polynucleotide phosphorylase (kindly donated by Dr. Maxine Singer), GDP, and [^{3}H]UDP. The base ratio of the product was 1:1 and the specific radioactivity was 10^7 cpm/μmol of nucleotide. Protein was determined by the method of Bücher (14).

Preparation of salt-washed ribosomes and crude initiation factor

Frozen *E. coli* Q13 cells (80 g) were ground with 160 g of powdered alumina and the resulting smooth paste was extracted with 240 ml of a solution containing 0.02 M Tris·HCl (pH 7.8), 0.03 M NH_4Cl, 0.01 M $Mg(OAc)_2$, 0.01 M 2-mercaptoethanol, and 5×10^{-4} M EDTA ("extraction buffer"). The resulting suspension was centrifuged twice at 15,000 rpm for 15 min, and the supernatant solution was then centrifuged for 3 hr at 50,000 rpm. The pellets were resuspended in 120 ml of extraction buffer which also contained 1.1 M NH_4Cl. After 1 hr at 4°C the ribosomal suspension was again subjected to low- and high-speed centrifugation. The

clear ribosomal wash fluid was fractionated with solid ammonium sulfate‡, and the material precipitating between 35 and 80% saturation was dissolved in 15 ml of 0.01 M potassium phosphate, pH 7.5, containing 10^{-3} M dithiothreitol, 5×10^{-4} M EDTA, and 10% glycerol. This fraction (crude initiation factor, approximately 250 mg of protein) was dialyzed overnight against the above phosphate buffer and quickly frozen.

The ribosomal pellets were resuspended in extraction buffer, treated with pancreatic DNase (2.5 μg/ml, Worthington, RNase-free), and then washed twice more in 1 M NH_4Cl. The washed ribosomes were then passed through a column of Sephadex G-200 (at least 20 volumes) equilibrated with 1.5 M NH_4Cl in extraction buffer. The eluted ribosomes were concentrated by high-speed centrifugation and suspended in a small volume of extraction buffer containing 10^{-3} M dithiothreitol instead of 2-mercaptoethanol. The ribosomes were stored at 0°C at a concentration of approximately 1000 A_{260}/ml, where they were stable for several weeks.

Assay of fMet-tRNA binding to ribosomes

Reaction mixtures (0.15 ml) contained 50 mM Tris·HCl (pH 7.8), 100 mM NH_4Cl, 10 mM $Mg(OAc)_2$, 4 mM 2-mercaptoethanol, 2 mM GTP, 2 A_{260} units of salt-washed ribosomes, initiation factors, 0.04 A_{260} unit of poly(U,G), and 1 A_{260} unit of [^{3}H]fMet-tRNA (containing approximately 40 pmol of methionine, of which 20 pmol was present in fMet-tRNA). When the crude initiation factor fraction was assayed, 10–20 μg of protein was added. The usual assay with purified factors employed 0.15 μg of FI, 0.15 μg of FII, and approximately 0.05 μg of FIII. After 10 min at 37°C, the reaction mixtures were diluted with 3 ml of cold reaction buffer and filtered through Millipore membranes, which were then washed with 6 ml of cold reaction buffer, dried, and counted. Alternatively, complex formation could also be measured by sucrose density gradient centrifugation analysis.

For the purification of factors, each fraction was assayed in the presence of the other two complementary factors and the Millipore technique was used. One unit of factor activity was defined as the amount that promotes the binding of 1 pmol of fMet-tRNA to ribosomes. Under the conditions described, the assay is linear with respect to FII up to a maximum of approximately 5 pmol of fMet-tRNA bound to ribosomes.

Preparation of [^{3}H]70S-ribosomes and assay for ribosome-dissociation activity

Although *E. coli* Q13 was used for the preparation of factors and salt-washed ribosomes, this strain proved to be unsuitable for preparation of 70S ribosomes. Q13 ribosomes were found to be extensively dissociated even after incubation of log phase cells at 15°C, a treatment reported to favor accumulation of 70S particles (9). 70S ribosomes could be obtained from *E. coli* MRE600 and therefore this strain was used. The method was essentially that described by Subramanian *et al.* (9) except that [^{3}H]uracil (1 μCi/ml) was included in the growth medium to provide greater sensitivity in the assay for dissociation.

Ribosome-dissociation activity was measured in reaction mixtures (0.125 ml) containing 10 mM Tris·HCl (pH 7.8), 50 mM KCl, 5 mM $Mg(OAc)_2$, 2×10^{-4} M dithiothreitol, 2 mM GTP, 0.3 A_{260} unit of [^{3}H]70S-ribosomes (containing approximately 60,000 cpm), and protein factor as indicated. After 15 min incubation at 30°C, 0.1-ml aliquots were layered onto chilled 5–20% sucrose gradients (5.3 ml) in reaction buffer and the tubes were centrifuged at 5°C for 85 min at 55,000 rpm in the SW65 rotor. Fractions (0.2 ml each) were collected, and their radioactivity content was determined in Bray's scintillation fluid. Ribosome dissociation was linear with respect to added factor up to approximately 70% dissociation and was expressed as picomoles of 30S particles formed.

Separation of factors on DEAE-cellulose

All buffers described in this section and the next contained 10^{-3} M DTT and 5×10^{-4} M EDTA and all operations were carried out at 0–4°C unless otherwise indicated. Crude initiation factor fraction (800 mg of protein) was applied to a column (3 × 30 cm) of DEAE-cellulose (Whatman DE23) equilibrated with 0.01 M potassium phosphate, pH 7.5. The column was washed with 600 ml of this buffer, followed by 600 ml of 0.01 M phosphate buffer, pH 7.5, containing 0.025 M NH_4Cl. A linear gradient of 1 liter total volume, from 0.025 to 0.15 M NH_4Cl, was then applied, followed by 500 ml of buffer containing 0.15 M NH_4Cl. Finally, another linear gradient of 1 liter total volume, from 0.15 to 0.35 M NH_4Cl, was applied. All eluting buffers contained 0.01 M potassium phosphate, pH 7.5. Fractions of 75 ml were collected, and 8 ml of glycerol was added to each. The fractions could be stored at 4°C for 1–2 days before being assayed.

FI activity appeared in the initial 0.01 M phosphate wash as a diffuse peak; FII activity was eluted toward the end of the first gradient; and FIII activity was eluted near the middle of the second gradient, often in two poorly resolved peaks. FI and FII were concentrated by adding solid ammonium sulfate to 85% saturation. FIII was concentrated by adding solid ammonium sulfate to 55% saturation. The resulting precipitates were dissolved in 0.05 M Tris·HCl (pH 7.8) containing 10% glycerol and then dialyzed against a large excess of this buffer for 6 hr. The solutions were quickly frozen and stored at −20°C. FI and FIII were further purified as described elsewhere (15).

Further purification of FII

The concentrated FII solution as described above was fractionated further (see Table 1) with solid ammonium sulfate: the activity was precipitated between 55 and 80% saturation. The resulting pellet was dissolved in 0.05 M Tris·HCl (pH 7.4)–0.05 M NH_4Cl–10% glycerol and dialyzed against a large excess of this buffer for 6 hr. The fraction was then applied to a 1.2 × 8 cm column of phosphocellulose (Whatman P11) equilibrated with 0.05 Tris·HCl, pH 7.4. The column was washed with 5 ml of this buffer, followed by 60 ml of 0.17 M potassium phosphate, pH 7.5. A linear gradient of 60 ml total volume, from 0.17 to 0.40 M potassium phosphate, pH 7.5, was then applied. FII activity appeared near the middle of the gradient. The active fractions were pooled and dialyzed for 6 hr against a large excess of a buffer containing 0.05 M Tris·HCl (pH 7.4)–0.05 M NH_4Cl–10% glycerol. The fraction was then applied to a 0.6 × 12 cm column of CM-Sephadex (Pharmacia C-50) previously equilibrated with 0.05 M Tris·HCl (pH 7.4)–0.05 M KCl. The column was washed with 6 ml of this buffer, followed by 12 ml of 0.05 M Tris·HCl

‡ All ammonium sulfate fractionations were carried out by adding solid ammonium sulfate to the desired concentration, stirring for 30 min, and centrifuging at 56,000 × *g* for 20 min.

(pH 7.4)–0.1 M KCl. A linear gradient of 30 ml total volume, from 0.1 to 0.5 M KCl, was then applied, followed by 10 ml of buffer containing 0.5 M KCl. All eluting buffers contained 0.05 M Tris·HCl, pH 7.4. FII eluted toward the end of the gradient.

This step did not increase the specific activity, but was employed to reduce possible FI contamination since FI is eluted from a similar column at a different position (15). The active fractions were pooled and concentrated about 2-fold by dialysis against 0.075 M Tris·HCl (pH 7.4)–0.075 M NH_4Cl–20% glycerol–20% polyethylene glycol (Carbowax 6000) followed by dialysis for 5 hr against a similar buffer lacking polyethylene glycol. The final preparation was stored at 0°C, where it was stable for at least six months. The purification of FII is summarized in Table 1.

TABLE 1. *Summary of purification of FII*

Fraction	Total units	Units/mg protein
1. DEAE-cellulose*	130,000	2,700
2. Ammonium sulfate	100,000	3,700
3. Phosphocellulose	69,000	44,900
4. CM-Sephadex	46,000	43,100

* The DEAE fraction was taken as the starting material since specific measurement of FII activity is difficult before separation of factors.

RESULTS

Polyacrylamide gel electrophoresis of FII

The FII preparation obtained as described above exhibited a single protein band on gel electrophoresis; the band migrated toward the cathode at pH 4.5 (Fig. 1*A*). No protein bands were observed migrating toward the anode at pH 8.5, which indicates that FII is a basic protein. The chromatographic behavior of FII on CM-Sephadex supports this conclusion.

SDS-polyacrylamide gel electrophoresis of FII

Purified FII preparations also were analyzed by sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (Fig. 1*B*). The single band observed provides further evidence of homogeneity and also indicates that FII is composed of a single type of polypeptide chain. The molecular weight of this chain is 21,000, as determined by its electrophoretic mobility (16). The marker proteins used in this determination, and their mobilities relative to FII were: ovalbumin, 0.56; chymotrypsinogen A, 0.89; myoglobin, 1.13, and cytochrome *c*, 1.33.

Role of FII in formation of the 70S initiation complex

The effect of FII on fMet-tRNA binding to ribosomes directed by the model messenger poly(U,G) was studied by sucrose gradient centrifugation. The results (Fig. 2) indicated that at 10 mM Mg^{++} fMet-tRNA binding to ribosomes promoted by poly(U,G) was almost completely dependent upon FII. The bound fMet-tRNA sedimented at 70 S, although the ribosome preparation used initially consisted largely of dissociated 30S and 50S subunits. No peak of radioactivity in the 30S region was detected in the presence or absence of FII. A small increase in the optical absorbance at 70 S was evident in the presence of FII, indicating the formation of the 70S complex from ribosomal subunits. In the absence of the other components of initiation, FII had no effect on the sedimentation profile of the ribosomes (not shown). The appearance of fMet-tRNA in the 70S region was also dependent upon poly(U,G), GTP, and FIII and was reduced approximately 2.5-fold by the omission of FI (Fig. 3). The methylene-bridged GTP analogue guanylyl(β,γ-methylene)diphosphonate (GMP-PCP) could not substitute for GTP (not shown).

In the absence of factors, fMet-tRNA, and GTP, [^{3}H]-poly(U,G) bound appreciably to 30S particles present in total salt-washed ribosomes, and bound (to a considerably lesser degree) to both 50S and 70S particles as well. However, a marked increase in 70S [^{3}H]poly(U,G) radioactivity was observed when the initiation factors, GTP, and unlabeled fMet-tRNA were added to the incubation mixture. The 70S regions obtained from several gradients in which various combinations of factors were employed are shown in Fig. 4. The inclusion of [^{3}H]poly(U,G) in a 70S peak was partially dependent upon FI, more markedly dependent upon FII, and almost completely dependent upon FIII. It was dependent as well on fMet-tRNA (Fig. 4) and on GTP (not shown). Leucyl-tRNA, or uncharged purified $tRNA^{fMet}$ could not substitute for fMet-tRNA nor could GMP-PCP substitute for GTP (not shown).

All three factors and GTP are therefore needed for maximum incorporation both of fMet-tRNA and poly(U,G) into the 70S chain initiation complex.

Ribosome dissociation activity of purified factors

It was of great interest to test our initiation factors for dissociating factor (DF) activity, particularly since both our FI and FII preparations appear to be homogeneous. The amounts of factors used in this experiment (Fig. 5) were in the same ratio as in a typical fMet-tRNA binding reaction but at 5 times the concentration. Under these conditions, FII exhibited extensive ribosome-dissociation activity, whereas FI and FIII were virtually inactive.

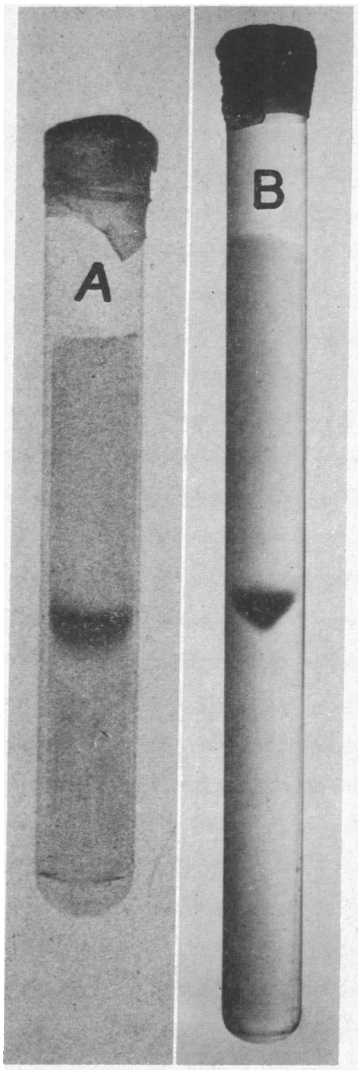
Cochromatography of FII and DF on CM-Sephadex

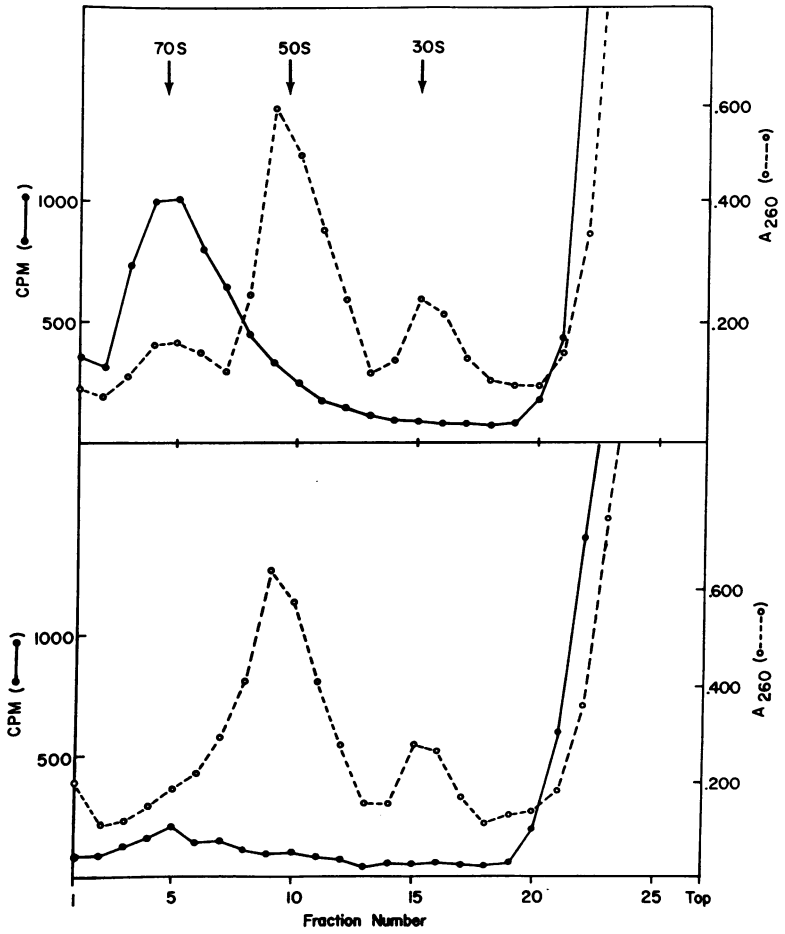
Although FII appeared homogeneous on gel electrophoresis, we could not rule out the possibility that a trace contaminant was responsible for the observed DF activity. To provide further evidence of the identity of FII and DF, successive fractions from the CM-Sephadex column (the last purification step) were assayed for both activities (Fig. 6). FII and DF activities appeared in the same region of the chromatogram and the ratios of the two activities in the active fractions were identical, within the limits of the assays.

Cosedimentation of FII and DF

Additional evidence that the fMet-tRNA binding activity and DF activity of FII are mediated by the same protein was obtained by glycerol density gradient centrifugation. Both activities cosedimented (Fig. 7), each active fraction again possessing the same ratio of the two activities.

This experiment also provided information about the native molecular weight of FII, since marker proteins were run in parallel gradients. The sedimentation constant thus obtained for FII was 2.2 S. Assuming that FII is a typical globular protein ($\bar{v}$ = 0.73; f/f_0 = 1.25), the native molecular weight based upon sedimentation is 20,000–25,000, in good agreement with the value of 21,000 obtained for the subunit molecular





(*Left*) FIG. 1. Polyacrylamide gel electrophoresis of FII. Purified FII (10 μg) was subjected to gel electrophoresis according to the methods described by Maizel (19). The gels were stained with Coomassie Blue and destained electrophoretically. *A*, native gel (7.5%), migration toward the cathode at pH 4.5. *B*, SDS–gel (10%), migration toward the anode at pH 7.0.

(*Right*) FIG. 2. Effect of FII on poly(U,G)-directed [^{3}H]fMet-tRNA binding to ribosomes. Reaction mixtures as described in *Materials and Methods* were incubated in the presence (*upper panel*) or absence (*lower panel*) of 0.15 μg of FII. After incubation, 0.1-ml aliquots were layered onto chilled 5–20% sucrose gradients (5.3 ml) in reaction buffer and centrifuged at 5°C for 85 min at 55,000 rpm in the SW65 rotor. Fractions of 0.2 ml were collected and their A_{260} was measured in microcuvettes. They were then transferred to scintillation vials, and their radioactivity was determined in Bray's scintillation fluid.

weight by SDS–gel electrophoresis. Therefore FII in its native state consists of a single polypeptide chain.

Other properties of FII

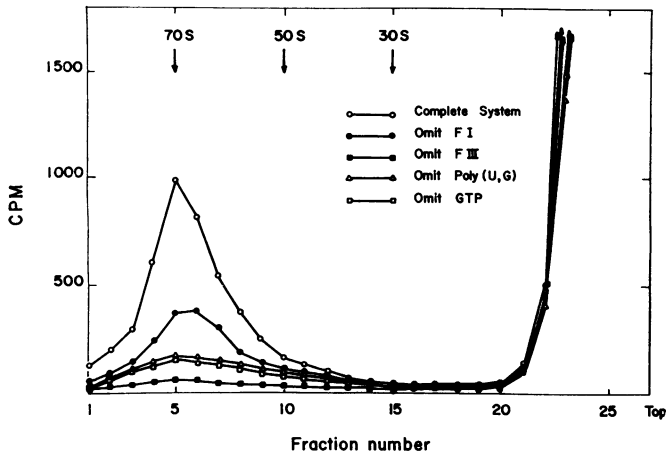
FII is a heat-stable protein, with the fMet-tRNA binding activity being inactivated only some 20% after 5 min at 90°C in the absence of glycerol. It was sensitive to trypsin. The fMet-tRNA binding activity of FII was not affected by treatment with 10^{-3} M *N*-ethylmaleimide or irreversibly affected by treatment with 10^{-3} M *p*-hydroxymercuribenzoate[§].

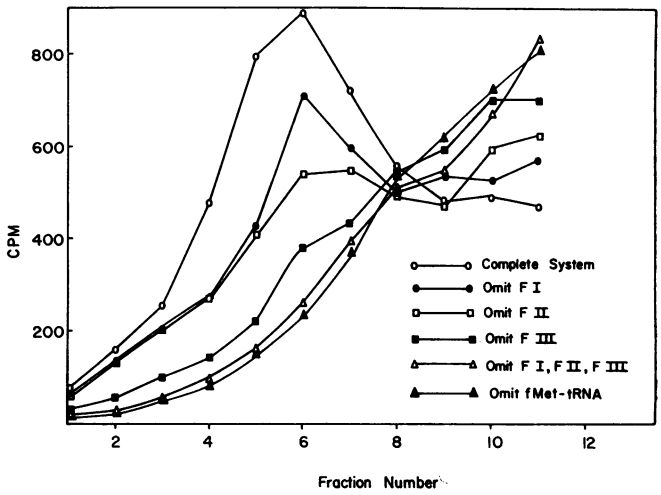
The ribosome-dissociation activity of FII showed similar resistance to heat and sulfhydryl reagents. In addition, DF activity was virtually abolished if GTP was omitted from the reaction mixture. GTP could be replaced, however, by the analogue GMP-PCP. Other nucleotides at 2 mM were less active than GTP as follows: ATP 80%, CTP 27%, and UTP 25%.

DISCUSSION

Evidence has been presented to show that purified FII (F_3, factor B) is required for the formation of the initiation complex of fMet-tRNA, poly(U,G), and the 70S ribosomal particle. Earlier conjectures (5–7) that this factor specifically recognizes 'natural messengers' now seem unlikely since poly(U,G) is a random polymer containing only two nucleotides. The presence, therefore, of specific natural initiation sequences in this polymer would be highly fortuitous, and indeed the actual sequences found near initiation sites in R17 RNA contain both C and A residues (17). Wahba *et al.* have also recently noted

[§] Reversible reaction with *p*-hydroxymercuribenzoate could not be ruled out because 2-mercaptoethanol had to be added to the reaction mixture to protect FIII, which is very sensitive to the reagent.





(*Left*) FIG. 3. Effect of other factors, GTP, and poly(U,G) on [^{3}H]fMet-tRNA binding to ribosomes. Reaction mixtures as described in *Materials and Methods* were analyzed for bound [^{3}H]fMet-tRNA as described in the legend to Fig. 2. Various components were omitted where indicated.

(*Right*) FIG. 4. Effect of purified initiation factors on the binding of [^{3}H]poly(U,G) to ribosomes in the 70S initiation complex. Reaction mixtures were as described in *Materials and Methods* except that 0.3 A_{260} unit of [^{3}H]poly(U,G), one A_{260} unit of salt-washed ribosomes, and one A_{260} unit of unlabeled fMet-tRNA were used. Centrifugation was carried out as described in the legend to Fig. 2; fractions of 0.2 ml were collected and counted directly in Bray's solution. For clarity, only the 70S region is shown.

(18) an effect of the factor corresponding to FII on systems employing synthetic messengers. Our observation that all three factors are required for the binding of fMet-tRNA in the initiation complex is in agreement with the results of Iwasaki *et al.* (5) using bacteriophage RNA as messenger, and in contrast to those of Revel *et al.* (6), who observed a minimal effect of factor B (FII, F_3) on fMet-tRNA binding using both AUG and T4 RNA messengers. Our studies of [^{3}H]-poly(U,G) binding to ribosomes indicate that all three factors, as well as GTP and fMet-tRNA, are required for maximum incorporation of mRNA into a 70S complex. These results are in contrast to reports suggesting a minimal role for FIII (F_2, factor C) in mRNA binding to a 70S complex (5, 8).

The ribosome-dissociation activity exhibited by FII is intriguing. We have presented evidence that DF activity is mediated by the protein that promotes formation of the 70S initiation complex. The relationship of the two activities is not yet clear. The salt-washed ribosomes used in normal fMet-tRNA binding experiments are already extensively dissociated under the normal reaction conditions, and there is no change in their sedimentation profile on incubation with FII alone at concentrations that promote fMet-tRNA binding. Therefore FII apparently does not bring about fMet-tRNA binding merely by generating 30S particles nonspecifically. Indeed, FII is still required for fMet-tRNA binding even if purified salt-washed 30S and 50S ribosomal subunits (which are added together, but which remain dissociated) are used. It is possible that a special active class of 30S particles is required, and that these are derived in an FII-mediated reaction from the small fraction of ribosomes present as 70S particles, in amounts too small to affect the gross sedimentation profile of the ribosomes. Alternatively, FII may act in the reverse direction during initiation, promoting the proper type of ribosome association. The 50S particle might conceivably react with the initial 30S complex to yield a 70S complex that is stable in the presence of FII and unstable in its absence. This possibility is under investigation. Finally, it may be that the dissociation activity

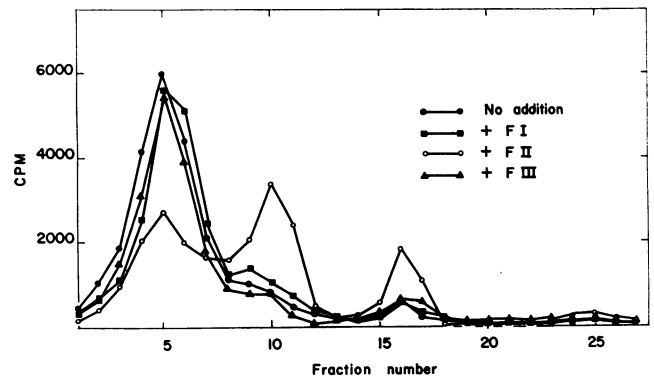


FIG. 5. Influence of factors on the dissociation of ribosomes. DF activity was assayed as described in *Materials and Methods*. Factor additions were 0.75 μg for FI and FII and 0.25 μg for FIII.

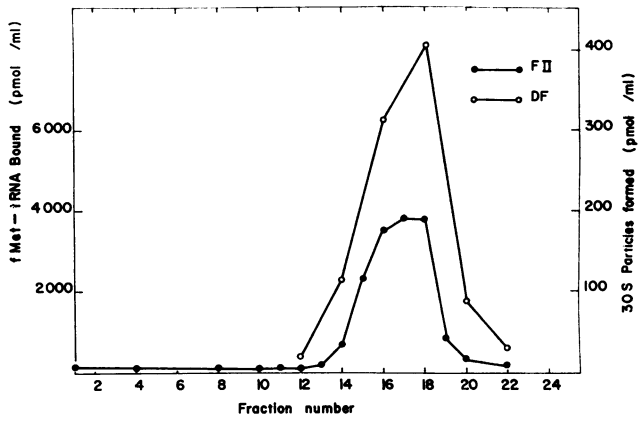


FIG. 6. Cochromatography of fMet-tRNA binding and DF activity of FII on CM-Sephadex. Fractions from CM-Sephadex step of purification were assayed for fMet-tRNA binding activity and for DF activity as described in *Materials and Methods*.

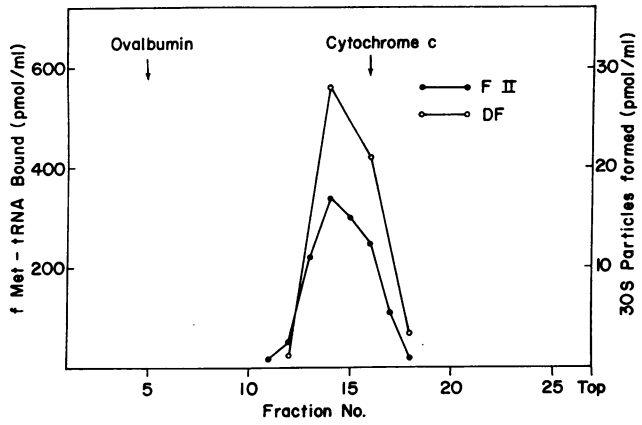


FIG. 7. Glycerol gradient centrifugation of fMet-tRNA binding and DF activities of FII. Purified FII was dialyzed against a buffer containing 0.05 M Tris·HCl (pH 7.4), 0.1 M NH_4Cl, 10^{-3} M dithiothreitol, 5×10^{-4} M EDTA, and 2% glycerol. 10 μg of dialyzed FII (0.1 ml) was applied to a gradient of 5–20% glycerol (5.3 ml) in the above buffer. Centrifugation was for 30 hr at 58,000 rpm at 5°C in the SW65 rotor. Fractions of 0.2 ml were collected and were assayed for fMet-tRNA binding activity and for DF activity as described in *Materials and Methods*. Ovalbumin and cytochrome *c* were run in parallel tubes, and were detected by optical absorbance at 280 and 410 nm, respectively.

of FII is physiologically independent of the binding activity, although residing on the same protein.

Miall *et al.* (12) have reported a polypeptide molecular weight of approximately 9,000 for a purified dissociation factor, in contrast to our value of 21,000. The reason for this difference is not yet clear, but it is possible that more than one type of dissociation factor may exist in bacterial cells.

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