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Dissociation of *Escherichia coli* Ribosomes. Role of Initiation Factors†

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ABSTRACT: The protein which promotes dissociation of *Escherichia coli* 70S ribosomes has been purified and identified as the initiation factor 3 (IF-3). The dissociating activity of this factor (dissociation factor, DF) is stimulated by the addition of initiation factor 1 (IF-1) and GTP. The DF activity is also enhanced by crude initiation factor 2 (IF-2) although it is not entirely certain whether the active component is the same as IF-2. The addition of fMet-tRNA to ribosomes maximally dissociated by DF in the presence of IF-1, IF-2, GTP, and poly(A-U-G) leads to the association of subunits and to the formation of the 70S initiation complex. Thus, the requirements for the maximum dissociation of 70S ribosomes and the formation of the 70S initiation complex appear to be similar.

A protein factor which dissociates ribosomes was first reported in *Escherichia* coli (Subramanian *et al.*, 1968) and subsequently in *Bacillus stearothermophilus* (Bade *et al.*, 1969), *Saccharomyces cerevisiae* (Pêtre, 1970), rat liver (Lawford *et al.*, 1971), and rabbit reticulocytes (Lubsen and Davis, 1972). Recently, several groups have reported that the *E. coli* dissociation factor (DF) is the same as the initiation factor 3 (IF-3) (Albrecht *et al.*, 1970; Sabol *et al.*, 1970; Subramanian and Davis, 1970; Dubnoff and Maitra, 1971; Sabol and Ochoa, 1971; Grunberg-Manago *et al.*, 1971; also see Davis, 1971). However, there is a lack of agreement on certain other points such as the stimulation of DF activity by GTP and stoichiometry between DF molecules and ribosomes dissociated. It is therefore uncertain whether the dissociating activity of IF-3 is an integral part of its role in the initiation of protein synthesis or merely a side effect *in vitro*. Moreover, we have previously presented evidence that much of the DF activity is associated with a protein fraction enriched with IF-1 (Miall *et al.*, 1970).

In order to explain these discrepancies, we have further studied the involvement of initiation factors in the dissociation of ribosomes. We report in this paper that the DF activity is in fact associated with the IF-3 fraction, but that its activity is stimulated by the addition of IF-1, IF-2, and GTP. In agreement with these findings Noll and Noll (1972) have recently reported that the dissociating activity of IF-3 is enhanced by IF-1.

Materials and Methods

Preparation of 70S Ribosomes. Ribosomes were prepared from *E. coli* B cells, harvested in mid-log phase after slow cooling, washed once with 0.5 M NH_4Cl and once with 1.0 M NH_4Cl, and resuspended in 10 mM Tris-HCl (pH 7.4), containing 5 mM Mg^{2+}, 50 mM KCl, and 6 mM β-mercaptoethanol.

Preparation of DF and Initiation Factors. E. coli ribosomes were washed once with 0.1 M NH_4Cl and then extracted with 1.0 M NH_4Cl. The 1.0 M NH_4Cl extract was fractionated by

† From The University of Alberta Cancer Research Unit, McEachern Laboratory, and the Department of Biochemistry, Edmonton, Alberta, Canada. *Received August 2, 1972.* This work was supported by funds from the National Cancer Institute of Canada and the Medical Research Council of Canada.

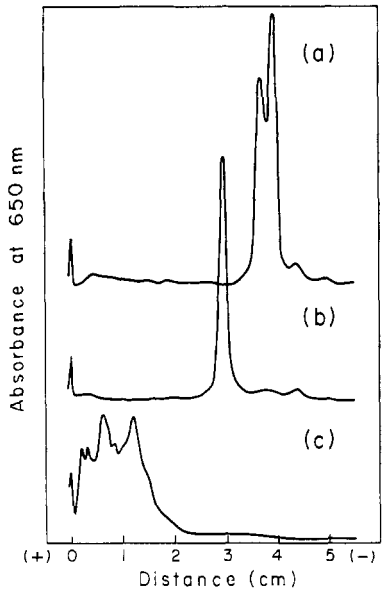

FIGURE 1: Densitometry of polyacrylamide gels of IF-1, IF-2, and DF. Electrophoresis was performed at pH 4.5 in 10% polyacrylamide gels in the presence of 8 M urea until the tracking dye, Pyronine, reached the end of the gel. The gels were stained with 1% Amido Black in 7.5% acetic acid. After the removal of excess dye the gels were scanned at A_{650} using a Gilford spectrophotometer with Model 2410 Linear transport. (a) IF-1, (b) DF, and (c) IF-2.

column chromatography on DEAE-cellulose and phosphocellulose as previously described (Miall *et al.*, 1970). The DF activity was eluted from the phosphocellulose column as a broad peak. Analysis by polyacrylamide gel electrophoresis showed that the active fractions contained both IF-1 and IF-3. The first one-third of the peak fraction which contained a relatively large portion of IF-1 was used to prepare IF-1 and the second two-thirds which contained a relatively large portion of IF-3 was used to prepare DF.

For the purification of IF-1, the sample was placed on a hydroxylapatite column (Bio-Gel HPT) equilibrated in 0.01 M potassium phosphate (pH 7.4) in the presence of 10% glycerol and 6 mM β-mercaptoethanol, and eluted with 0.075, 0.15, and 0.3 M potassium phosphate. The fraction eluted with 0.15 M potassium phosphate was used as purified IF-1.

Upon electrophoresis in polyacrylamide gel in the presence of 8 M urea, this preparation exhibited a closely migrating double band (Figure 1a). In the absence of urea only a single band was observed. We do not know the significance of this phenomenon.

For the purification of DF the sample was placed on a column as above and was eluted with 200 ml of 0.01–0.25 M potassium phosphate gradient (pH 7.4) in the presence of 10% glycerol and 6 mM β-mercaptoethanol. The DF activity was eluted in two separate peak fractions (Figure 2).

The first fraction, eluted at 40–90 mM potassium phosphate, contained 40% of the DF activity recovered. Analysis on polyacrylamide gels showed about 80% of the total protein in the sample was IF-1 and 5–10% IF-3. On the other hand, the second DF fraction, eluted at 0.1–0.15 M potassium phosphate, contained 7–10% of IF-1, 85% of IF-3, and 60% of the DF activity recovered.

The second DF fraction was further purified by rechromatography on hydroxylapatite. The loaded column was eluted batchwise with 0.15, 0.2, 0.25, and 0.3 M potassium phosphate. The majority of the DF activity (70%) was in the

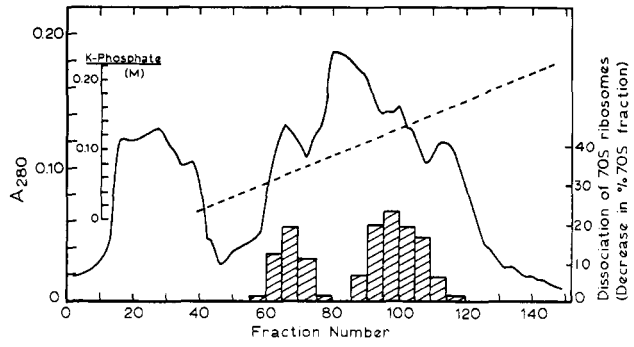

FIGURE 2: Chromatography of DF on hydroxylapatite. A DF preparation partially purified by chromatography on phosphocellulose was further purified by hydroxylapatite chromatography as described in Materials and Methods. ———, A_{280}; – – –, potassium phosphate concentration. Hatched areas show the ribosome dissociating activity of 20-μl samples from each column fraction.

fraction eluted with 0.25 M potassium phosphate. On polyacrylamide gel electrophoresis in the presence of 8 M urea this DF preparation exhibited a major band (95%) and several minor bands (5%) (Figure 1b). The molecular weight of the major band, as estimated by sodium dodecyl sulfate gel electrophoresis (Dunker and Rueckert, 1969), was 21,000 which is the same as that of IF-3 (Sabol *et al.*, 1970; Dubnoff and Maitra, 1971).

We assumed that the major band represented DF and IF-3 in accord with the published data that DF is the same as IF-3. If, instead, a minor band had the DF activity the number of ribosomes dissociated by one molecule of DF would far exceed that expected from the stoichiometric relationship (see Discussion).

IF-2 was prepared by DEAE-cellulose chromatography of the 1 M NH_4Cl ribosomal extract according to Lucas-Lenard and Lipmann (1966), and was further purified by $(NH_4)_2SO_4$ precipitation, collecting the fraction precipitated between 33 and 55% saturation. On polyacrylamide gel electrophoresis the IF-2 preparation showed several protein bands, primarily near the origin (Figure 1c). However, it is evident that there is little contamination by IF-1 and IF-3 which was supported by the binding assay reported in Table I.

Preparation of [^{3}H]fMet-tRNA. [^{3}H]fMet-tRNA was prepared by charging stripped *E. coli* B tRNA with [^{3}H]methio-

TABLE I: Binding of [^{3}H]fMet-tRNA to Ribosomes in the Presence of Initiation Factors.[a]

Factors Added	[^{3}H]fMet-tRNA Bound (pmoles)
IF-1	0.20
IF-2	0.15
DF	0.19
IF-1 + IF-2	0.23
IF-1 + DF	0.21
IF-2 + DF	0.33
IF-1 + IF-2 + DF	1.64

[a] Assay conditions are described in Materials and Methods. All values were corrected for binding in the absence of added factors.

TABLE II: Stimulation of DF Activity by IF-1 and IF-2.[a]

Factors Added	70S Ribosomes	
	% of Control	% Decrease
None	100.0	
DF	81.5	18.5
IF-1	95.0	5.0
IF-2	97.5	2.5
DF + IF-1	64.3	35.7
DF + IF-2	67.9	32.1
DF + IF-1 + IF-2	65.2	34.8

[a] The assay conditions for dissociation of 70S ribosomes are described in Materials and Methods. The amounts of each factor added per reaction mixture (0.35 ml) were: DF, 1.1 μg; IF-1, 4 μg; IF-2, 50 μg. The reaction mixtures were incubated for 15 min at 37°. The 70S ribosomes in the control sample were 67.5% of the total uv-absorbing material in the sample.

nine in the presence of N^5-formyltetrahydrofolate (Ca-Leucovorin, American Cyanamide Co., Pearl River, N. Y.), using a crude *E. coli* supernatant fraction (Marcker, 1965).

Assay of Initiation Complex Formation. BINDING OF [^{3}H]-fMet-tRNA TO RIBOSOMES. The binding of [^{3}H]fMet-tRNA to ribosomes was assayed according to Wahba *et al.* (1969) except that poly(A-U-G) was used instead of A-U-G triplet. The incubation mixture (0.29 ml) contained 50 mM Tris-HCl (pH 7.4), 50 mM NH_4Cl, 5 mM Mg^{2+}, 6 mM β-mercaptoethanol, 150 pmoles of [^{3}H]fMet-tRNA, 10 μg of poly(A-U-G), 0.35 mM GTP, 2.0 A_{260} units of ribosomes, 8 μg of IF-1, 26 μg of IF-2, and 1.8 μg of DF. Mixtures were incubated at 25° for 10 min and then filtered through Millipore filters as described by Nirenberg and Leder (1964).

This assay system is dependent upon poly(A-U-G), GTP as well as all the three initiation factors. The initiation complex formed at 25° is stable and the subsequent cooling of the sample to 0° does not alter the amount of [^{3}H]fMet-tRNA bound at 25° (manuscript submitted for publication).

Assay of Ribosome Dissociation. The reaction mixture (0.35 ml) comtained 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 50 mM KCl, 6 mM β-mercaptoethanol, and 0.5 A_{260} unit of 70S ribosomes. Initiation factors (4 μg of IF-1 and 50 μg of IF-2), 1.1 μg of DF, 0.3 mM GTP, 5 μg of poly(A-U-G), and 38 pmoles of [^{3}H]fMet-tRNA were added as indicated. The samples were incubated at 37° for 15 min and then layered on to 5–20% sucrose gradients in the same buffer as the sample without β-mercaptoethanol and centrifuged at 48,000 rpm for 45 min in the Spinco SW50.1 rotor. The gradients were analyzed from the top using a flow cell attached to a Gilford spectrophotometer. Fractions of 0.25 ml each were collected and [^{3}H]fMet-tRNA bound to ribosomes was determined according to Nirenberg and Leder (1964). The amount of 30S, 50S, and 70S fractions was measured by using a planimeter. The overall assay of ribosome dissociation was reproducible with an error of less than 3%.

Chemicals. [^{3}H]Methionine (3.3 Ci/mmole) was purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y., *E. coli coli* B tRNA from General Biochemicals, Chagrin Falls, Ohio; poly(A-U-G) (A:U:G = 1.91:0.63:1.00) and *E. coli* B cells from Miles Laboratories (Kankakee, Ill.).

Results

Analysis of Purified DF for Initiation Activity. The purified DF preparation was analyzed for possible initiation factor activity by measuring the stimulation of the binding of [^{3}H]-fMet-tRNA to ribosomes (Table I). The results show that, in the presence of IF-1 and IF-2, the addition of DF stimulated the binding about 5-fold. This, together with the behavior or polyacrylamide gels (see Materials and Methods and Figure 1b), indicates that DF is the same as IF-3. It is also clear from the results that there was little cross-contamination among the three factor preparations employed.

Stimulation of DF Activity by IF-1 and IF-2. Several observations made during the purification of DF suggested that IF-1 stimulated the DF activity of IF-3. For example, in our previous study in which ribosomes were washed first with 0.5 M NH_4Cl and then with 1.0 M NH_4Cl, we found that about 80% of the DF activity was associated with the 0.5 M NH_4Cl extract and only about 20% with the 1.0 M NH_4Cl extract. However, the 0.5 M NH_4Cl extract contained mainly IF-1 and IF-2 and the 1 M NH_4Cl extract contained mainly IF-3 (Iwasaki *et al.*, 1968). Thus the DF activity was not directly related to the amount of IF-3 in the preparation.

In the present study, the initial hydroxylapatite chromatography yielded two DF fractions (Figure 2) which contained both IF-1 and IF-3 proteins in different amounts. Again the DF activity of these fractions was not proportional to the amounts of IF-3 (see Materials and Methods). Thus it would appear that the presence of IF-1 plays an important role in the expression of DF activity of IF-3.

We tested, therefore, the effect of IF-1 and IF-2 on the DF activity of IF-3 (Table II). The results show that both IF-1 and IF-2 stimulated the DF activity. The degree of stimulation ranged from 1.5- to 3-fold depending upon the DF preparation and the concentration of IF-1 and IF-2. Except for a few cases, the simultaneous addition of these two factors did not increase the stimulation appreciably.

We found that if an IF-1 preparation lost the activity to stimulate binding of [^{3}H]fMet-tRNA to ribosomes it also lost the activity to stimulate DF activity. However, this was not necessarily the case with IF-2. We observed that an IF-2 sample, prepared in the presence of 6 M urea and inactive in the [^{3}H]fMet-tRNA binding assay, stimulated ribosomal dissociation to the same extent as did a functional IF-2 preparation.

Effect of GTP, Poly(A-U-G), and fMet-tRNA on Dissociation of Ribosomes. The effect of GTP, poly(A-U-G) and fMet-tRNA on the DF activity was examined in the presence of IF-1 and IF-2. In one experiment, GTP, poly(A-U-G) and [^{3}H]fMet-tRNA were added to ribosomes with the initiation factors and incubated at 37° for 15 min (Simultaneous Addition in Table III). In the other, ribosomes were preincubated with the initiation factors at 37° for 10 min, GTP, poly-(A-U-G), and [^{3}H]fMet-tRNA were then added and the incubation was continued for an additional 5 min (Delayed Addition in Table III). In both cases the dissociation was increased by the addition of GTP or GTP + poly(A-U-G) although the increase was greater in the delayed addition than in the simultaneous addition. The reason for this difference is not clear at present. The effect of GTP on the Mg^{2+} concentration, if any, should be small under the experimental conditions (*i.e.*, the maximum change would be a decrease from 5 to 4.7 mM). Moreover such an effect should be independent of the time of addition of GTP and thus the chelation of Mg^{2+} by GTP as a major cause for the increased dissociation may be ruled out.

TABLE III: Effect of GTP, Poly(A-U-G), and fMet-tRNA on the Dissociation of 70S Ribosomes.[a]

Additions		70S Ribosomes (% of Control)	
Initiation Factors	Others	Simultaneous Addition	Delayed Addition
None	None	100.0	100.0
IF-1, IF-2, DF	None	72.5	68.2
IF-1, IF-2, DF	GTP	59.2	41.9
IF-1, IF-2, DF	GTP + poly(A-U-G)	59.6	40.0
IF-1, IF-2, DF	GTP + poly(A-U-G) + [^{3}H]fMet-tRNA	66.0	45.7

[a] The amounts of initiation factors used were the same as described in Table II. Other compounds used were: GTP, 0.3 mM; poly(A-U-G), 5 μg; and [^{3}H]fMet-tRNA, 38 pmoles. In simultaneous addition, GTP, poly(A-U-G), and [^{3}H]fMet-tRNA were added to ribosomes at zero time with initiation factors and incubated for 15 min at 37°. In delayed addition, ribosomes were incubated with initiation factors for 10 min at 37° and the other compounds were added and the incubation was continued for an additional 5 min at 37°. The reaction mixtures were then centrifuged through sucrose gradients as described in Materials and Methods. Fractions of 0.25 ml each were collected from the gradient and [^{3}H]fMet-tRNA bound to ribosomes was determined by the Millipore filter method. The amounts of 70S ribosomes in the controls were 72.6 and 76.8% of the uv-absorbing material for simultaneous addition and delayed addition, respectively.

TABLE IV: Association of Ribosomal Subunits by [^{3}H]fMet-tRNA and the Effect of GTP and Poly(A-U-G).[a]

Sample	Additions		70S Ribosomes (% of Control)	[^{3}H]-fMet-tRNA Bound to 70S Fraction (cpm)
	First Incubn	Second Incubn		
1	None	None	100.0	
2	IF-1, IF-2, DF	None	72.2	
3	IF-1, IF-2, DF	[^{3}H]fMet-tRNA	72.3	57
4	IF-1, IF-2, DF, GTP, poly-(A-U-G)	None	59.0	
5	IF-1, IF-2, DF, GTP, poly-(A-U-G)	[^{3}H]fMet-tRNA	66.3	151
6	IF-1, IF-2, DF	GTP, poly(A-U-G), [^{3}H]fMet-tRNA		181
7	IF-2, DF	GTP, poly(A-U-G), [^{3}H]fMet-tRNA		64
8	IF-1, DF	GTP, poly(A-U-G), [^{3}H]fMet-tRNA		5
9	IF-1, IF-2	GTP, poly(A-U-G), [^{3}H]fMet-tRNA		24

[a] The amounts of initiation factors and other compounds used were the same as described in Table III. The first incubation was for 10 min at 37° and the second incubation for 5 min at 37°. The amount of 70S ribosomes in the control was 72.5% of the total uv-absorbing material in the sample.

The addition of [^{3}H]fMet-tRNA, on the other hand, was found to reduce the amount of ribosomal dissociation (by 6%) in the presence of GTP and poly(A-U-G). This effect of [^{3}H]fMet-tRNA could be due to either inhibition of ribosomal dissociation or reassociation of subunits. In order to distinguish between these possibilities we examined the effect of [^{3}H]fMet-tRNA added to ribosomes which had been dissociated maximally by incubation with initiation factors, GTP and poly(A-U-G) (Table IV). It was found that such a delayed addition of [^{3}H]fMet-tRNA also resulted in 6% increase in the 70S fraction (*cf.* samples 4 and 5 in Table IV). Thus we conclude that the reduced dissociation of 70S particles in the presence of [^{3}H]fMet-tRNA is due to reassociation of subunits. As also shown in Table IV (*cf.* samples 2 and 3) the presence of GTP and poly(A-U-G) is essential for this reassociation. In their absence [^{3}H]fMet-tRNA has no effect in either association or dissociation.

The last column of Table IV shows the amount of [^{3}H]-fMet-tRNA bound to 70S particles (*i.e.*, the formation of the 70S initiation complex) as assayed by sucrose density gradient centrifugation. The maximum binding required the presence of GTP and poly(A-U-G) and all the three initiation factors (samples 6–9 in Table IV; Iwasaki *et al.*, 1968; Kondo *et al.*, 1968).

Discussion

The results presented in this paper show that although IF-3 is primarily responsible for the dissociation of 70S ribosomes its DF activity is enhanced by the presence of IF-1 and GTP. The effect of IF-2 is inconclusive since we found that an IF-2 preparation which was inactive in the [^{3}H]fMet-tRNA binding assay retained the stimulatory activity in the DF assay. However, the involvement of at least two initiation factors in the ribosomal dissociation suggests that the dissociation may be an integral initial step of the initiation mechanism which involves 70S ribosomes. This is supported by the results presented in Table IV which show that the addition of fMet-tRNA to the dissociated ribosomes in the presence of all initiation factors, GTP, and poly(A-U-G) resulted in the formation of 70S initiation complex through association of subunits in the presence of DF. Similar results have been reported by others (Sabol *et al.*, 1970; Sabol and Ochoa, 1971; Dubnoff and Maitra, 1971; Davis, 1971).

The stimulation of DF activity by other factors may explain several previous observations which are seemingly contradictory. For instance, although a model in which each DF molecule dissociates one 70S ribosome *in vivo* has been proposed, assays *in vitro* have never achieved the stoichiometry. With highly purified DF and at 5 mM Mg^{2+} over 30 moles of DF is shown to be required to dissociate 1 mole of 70S ribosomes (Subramanian and Davis, 1970). Also using a highly purified DF preparation but in the presence of 2 mM GTP (*cf.* 0.3 mM in this study) a DF: ribosome ratio of less than 10 has been reported (Dubnoff and Maitra, 1971). How-

ever, little DF activity was found in the absence of GTP and the possibility that the effect of GTP is largely due to chelation of Mg^{2+} cannot be ruled out. Only with less pure DF preparations has near stoichiometry (DF:70S ribosomes = 2:1) been reported (Albrecht *et al.*, 1970). In the present study the DF:ribosome ratio has been reduced to 7 at 5 mM Mg^{2+} in the presence of other initiation factors, GTP, and poly(A-U-G). It thus appears that the DF activity depends very much upon the purity of the preparation, in particular, with respect to other initiation factors and GTP. It may also be possible that the IF-3 protein purified 100% might lack intrinsic DF activity. It has been reported that when IF-3 is further fractionated into several subclasses with respect to the specificity to mRNA only certain IF-3 subfractions exhibit DF activity (Grunberg-Manago, 1971; Vermeer *et al.*, 1971). In the light of the present results the possibility exists that after subfractionation some IF-3 species are highly pure but others are still associated with IF-1 or IF-2 and thus exhibit DF activity.

Acknowledgments

We thank Mrs. M. J. Gillespie and Miss M. Battell for technical assistance.

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