THE EFFECTS OF INITIATION FACTORS IF-1 AND IF-3 ON THE DISSOCIATION OF *ESCHERICHIA COLI* 70 S RIBOSOMES

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1. Introduction

It is well established that a dynamic equilibrium exists between 70 S ribosomes and 50 S and 30 S subunits [1–4]. Several groups [5–12] have shown that IF-3 binds to the 30 S subunit, preventing association of the subunits, thereby shifting the dynamic equilibrium toward dissociation. Further work has indicated that the dissociation of 70 S ribosomes mediated by IF-3 [9,12–15] is enhanced by IF-1, however, the mechanism by which IF-1 functions in this process is still unclear. The primary method used to study ribosomal dissociation has been sucrose density gradient centrifugation [5–11,13–15]. The data obtained by this method are sometimes difficult to interpret because of the artifactual association and dissociation of the ribosomes which occur during the course of the centrifugation [1–4] More recently, light scattering has been employed to measure ribosomal dissociation [12].

In this investigation we have studied the effects of IF-1 and IF-3 on the dissociation of 70 S ribosomes using a simple, two-step assay procedure which is based on the observations in [16,17].

(i) Starting with 70 S ribosomes, maximal binding of fMet-tRNA directed by AUG is obtained at 25°C in the presence of IF-1, IF-2, IF-3 and GTP, however, very little binding is observed at 0°C.
(ii) Dissociation of 70 S ribosomes mediated by IF-3 and IF-1 occurs rapidly at 25°C but slowly at 0°C
(iii) Starting with isolated 30 S subunits, AUG-directed binding of fMet-tRNA occurs rapidly at 0°C in the presence of IF-2 and GTP.

In the first step of the two-step assay, 70 S ribosomes are incubated at 25°C with or without IF-1 and IF-3. In the second step, the temperature is lowered to 0°C, IF-2, fMet-tRNA, GTP and AUG are added, and the reaction mixture is incubated at 0°C The amount of fMet-tRNA bound to the ribosomes is determined by the Millipore filter technique [18] and is a measure of the amount of dissociation occurring during the first step.

Using this two-step assay procedure, we find that IF-1 enhances both the rate and the extent of ribosomal dissociation obtained with IF-3, and the degree of enhancement observed with IF-1 is dependent upon the concentrations of Mg^{2+} and NH_4^+.

2. Experimental procedures

2.1. *Initiation factors*

IF-1, IF-2 and IF-3 were isolated from the 1 M NH_4Cl wash of *E coli* Q13 or MRE 600 ribosomes by a modification of the procedures in [17,19] The 0–80% ammonium sulfate precipitate obtained from the ribosomal wash was applied to a DEAE-cellulose column in buffer D (50 mM Tris·HCl (pH 7.5), 0.5 mM dithiothreitol, 1 mM EDTA and 10% glycerol) containing 0.05 M NH_4Cl Both IF-1 and IF-3 were recovered in the effluent, IF-2 was retained by the column and was eluted with buffer D containing 0.25 M NH_4Cl. IF-1 and IF-3 were separated by chromatography on phosphocellulose using a linear gradient from 0.1–0.6 M NH_4Cl in buffer D. IF-1 eluted from the column at ~0.2 M and IF-3 at ~0.4 M NH_4Cl. IF-1 and IF-3 were further purified by chromatography on Sephadex G-50 and G-75, respectively. IF-2 was

purified by chromatography on phosphocellulose as in [17]. Protein concentrations were determined by a modification of the Folin–Lowry procedure [20]. SDS–gel electrophoresis indicated that the factors were > 90% pure.

2.2. *Ribosomes*

Salt-washed ribosomes were prepared from *E. coli* W or MRE 600 cells as in [21]. Ribosomes, 70 S, free of 30 S subunits, were isolated by centrifugation through a 10–30% (w/v) linear sucrose gradient in buffer Z (20 mM Tris·HCl (pH 7 7), 0.1 M NH_4Cl, 1 mM dithiothreitol and 10 mM $MgCl_2$) for 3.5 h at 48 000 rev /min at 4°C in a Beckman Ti-14 zonal rotor. The peak fractions in the 70 S region were pooled and the ribosomes were collected by centrifugation at 60 000 rev./min for 12 h at 4°C in a Beckman Ti-60 rotor. The 70 S ribosomes were then resuspended at 50 mg/ml in buffer C (10 mM Tris·HCl (pH 7 7), 10 mM $MgCl_2$, 50 mM $MgCl_2$, 50 mM NH_4Cl and 1 mM dithiothreitol) and were stored in small aliquots at −70°C

2 3. *Two-step dissociation assay*

The first incubation mixture contained in 0.075 ml: 45 mM Tris HCl (pH 7.7), 80 mM NH_4Cl, 5 mM $MgCl_2$, 5 mM dithiothreitol, 10 μg bovine serum albumin, 0 72 A_{260} units of 70 S ribosomes (~18 pmol), and IF-3 and IF-1, as indicated. The reaction mixture was incubated for 5 min at 25°C and then placed in an ice bath. For the second incubation, the volume was increased to 0.15 ml, and 2 μg IF-2, 0.2 A_{260} units of AUG, 45 nmol GTP and 15 pmol f[^{3}H]Met-tRNA (400 cpm/pmol), prepared as in [18,19], were added The final concentrations of the other components of the reaction mixture were as follows: 50 mM Tris HCl (pH 7.7), 5 mM $MgCl_2$, 100 mM NH_4Cl and 5 mM dithiothreitol. After 5 min incubation at 0°C, the amount of [^{3}H]Met-tRNA bound to the ribosomes was determined by the Millipore filter technique as in [18]

2.4. *Sucrose gradient analysis*

To measure ribosomal dissociation by the sucrose density gradient method, 70 S ribosomes (~18 pmol) were incubated as above for the first step of the two-step assay procedure. After 5 min at 25°C, the reaction mixture was placed in an ice bath and 0.075 ml 50 mM Tris·HCl (pH 7 7) containing 5 mM $MgCl_2$, 100 mM NH_4Cl, and 1 mM dithiothreitol was added. The entire sample (0.15 ml) was layered on a 14 ml linear 10–30% (w/v) sucrose gradient in buffer Z containing 5 mM $MgCl_2$ and was centrifuged at 20 000 rev./min for 18 h at 4°C in a Beckman SW-40 rotor. After centrifugation, the absorbance profile at 254 nm was obtained by the use of an ISCO gradient analyzer and recorder. The areas under the peaks in the 30 S, 50 S and 70 S regions were measured with a planimeter. The % dissociation was determined by dividing the sum of the areas under the 30 S and 50 S peaks by the total area under all three peaks. The no. pmol of dissociated ribosomes was calculated from an average of the % dissociation obtained for duplicate samples.

3. Results

As shown in table 1, very little binding of fMet-tRNA was obtained when 70 S ribosomes were incubated at 25°C for 5 min in the absence of IF-1 and IF-3 and then incubated for 5 min at 0°C in the presence of a stoichiometric amount of IF-2. No significant increase in the amount of fMet-tRNA bound was observed when IF-1, IF-3, or IF-1 and IF-3 were present only during the second incubation, nor was a significant increase obtained when IF-1 alone was added to the

Table 1
The amount of fMet-tRNA bound to ribosomes in response to IF-3 and IF-1 added to the first or second step of the two-step binding assay

1st inc 5 min 25°C		2nd inc 5 min 0°C		fMet-tRNA bound (pmol)
IF-1	IF-3	IF-1	IF-3	
–	–	–	–	0 5
–	–	+	–	0.6
–	–	–	+	0 5
–	–	+	+	0 7
+	–	–	–	0 6
–	+	–	–	2 0
–	+	+	–	2 1
+	–	–	+	1.2
+	+	–	–	7 7

The reactions mixtures were supplemented with 1 μg IF-3 and 2 5 μg IF-1 as indicated. In all of the above the second incubation mixture contained 2.0 μg IF-2

first incubation mixture. However, addition of IF-3 to the first incubation mixture produced a 3–4-fold increase in the amount of fMet-tRNA bound. The amount bound was not significantly greater when IF-1 was added to the second incubation mixture. If IF-1, as well as IF-3, were present during the first incubation, the amount of fMet-tRNA bound was 3–4-times greater than that obtained with IF-3 alone.

The data in fig.1A show that about 2 pmol fMet-tRNA were bound to the ribosomes when 1 μg IF-3 was added, and that the binding of fMet-tRNA did not increase when the amount of IF-3 was increased to 4 μg. Results obtained from a large number of experiments showed that the maximal amount of fMet-tRNA bound without IF-1 under this specific set of conditions, i.e., 5 mM Mg^{2+} and 80 mM NH_4^+, was 1.5–3 pmol/18 pmol ribosomes. Addition of IF-1, as well as IF-3, to the first incubation mixture increased the amount of fMet-tRNA bound to ~10 pmol. The amount of IF-1 necessary to obtain maximal stimulation was 1 μg (fig.1B). In separate experiments it was found that the amount of fMet-tRNA bound in the presence of both IF-3 and IF-1 ranged from 7–10 pmol/18 pmol ribosomes, varying from one preparation of ribosomes to another. These data indicate that only 30–50% of the ribosomes are

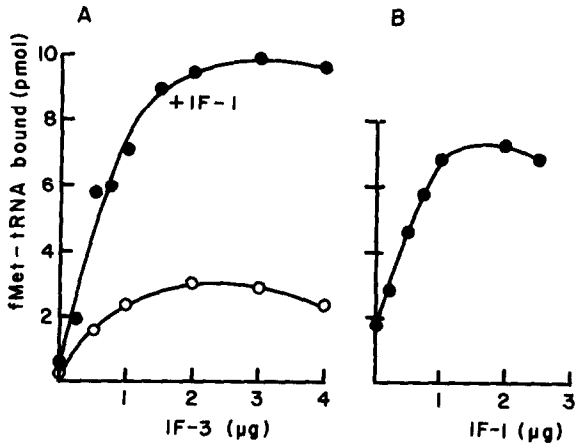

Fig 1. fMet-tRNA bound to ribosomes in response to IF-3 and IF-1. In (A) the first incubation mixture contained IF-3, in the amounts indicated, and either no IF-1 (–○–○–) or 2.5 μg IF-1 (–●–●–). In (B) the first incubation mixture contained 1 μg IF-3 and IF-1 in the amounts indicated. The amount of fMet-tRNA bound was determined as in section 2

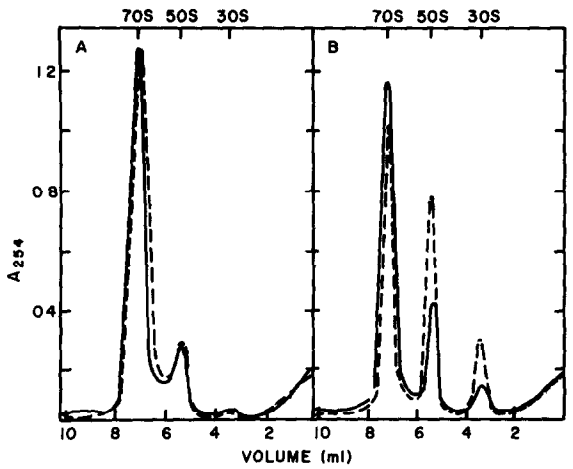

Fig 2 The effects of IF-3 and IF-1 on the dissociation of 70 S ribosomes. 70 S ribosomes (~18 pmol) were incubated for 5 min at 25°C in the presence or absence of IF-1 and IF-3 as indicated (A), no factors (———) and 2 5 μg IF-1 (– – – – –), (B), 1 μg IF-3 (———) and 1 μg IF-3 and 2 5 μg IF-1 (– – – – –) Sucrose density gradient analysis was carried out as in section 2

capable of binding fMet-tRNA and are in agreement with the findings of other investigators [9,11,13]. It is of interest to note that the amount of IF-3 necessary to obtain half-maximal binding in the absence of IF-1 and in the presence of IF-1 was ~0.5 μg or 22 pmol in both cases. When the number of ribosomes was increased 2-fold (36 pmol), the amount of IF-3 required for half-maximal binding increased 2-fold and the amount of IF-1 required also increased proportionately.

To confirm that the amount of binding obtained in response to the addition of IF-3, or IF-3 and IF-1, in the first step of the assay was due to an increase in the amount of dissociation that occurred during the first incubation, 70 S ribosomes were incubated under the identical conditions used for the first step of the assay. The second step was omitted, and the reaction mixtures were analyzed by the sucrose density gradient method. As shown in fig.2A, a small amount of the ribosomes (~10%) dissociated under these conditions in the absence of IF-1 and IF-3, and no detectable increase in dissociation was obtained when the ribosomes were incubated with IF-1 alone. An increase in the amount of 30 S and 50 S subunits was obtained with IF-3 alone, and a still greater increase was

Table 2
Comparison of the two-step binding assay and the sucrose density gradient method of measuring dissociation of 70 S ribosomes mediated by IF-3 and IF-1

Factors added	Two-step binding assay		Sucrose density gradient	
	fMet-tRNA bound (pmol)		70 S ribosomes dissociated (pmol)	
None	0.5	–	2.0	–
IF-1, 2.5 μg	0.6	0.1	2.1	0 1
IF-3, 1.0 μg	2 0	1.5	3.8	1 8
IF-1, 2.5 μg + IF-3, 1.0 μg	7.2	6.7	8.1	6.1

obtained when both IF-3 and IF-1 were present (fig.2B). Increased amounts of IF-3 alone ($\leqslant 4$ μg) did not increase the degree of dissociation significantly above that obtained with 1 μg IF-3 alone.

A comparison of the results obtained by the two procedures is given in table 2. In the absence of IF-3 and IF-1 the amount of dissociation observed on sucrose gradients was greater than the amount of fMet-tRNA bound in the two-step assay procedure. When the values obtained in the absence of factors were subtracted from the values obtained in the presence of the factors, a close correlation between pmol fMet-tRNA bound and pmol ribosomes dissociated was obtained. With either procedure a 3–4-fold stimulation was observed when IF-1 was added in the presence of IF-3. Thus, the two-step assay procedure provides a rapid and simple means of studying ribosomal dissociation

Using the two-step binding assay, the rate at which factor-mediated ribosomal dissociation occurred was determined. As described in the legend to fig.3, the first incubation time was varied from 0–20 min, while the second step incubation time was held constant at 5 min. When IF-3 and IF-1 were present in the first incubation mixture, the amount of fMet-tRNA bound increased with the first incubation time up to 5 min, at which time maximal binding (~8 pmol) was obtained. When IF-3 alone was present in the first incubation mixture, the amount of fMet-tRNA bound also increased with time up to 5 min. However, the amount bound after 5 min was only 2–3 pmol, and no further increase in the amount of fMet-tRNA

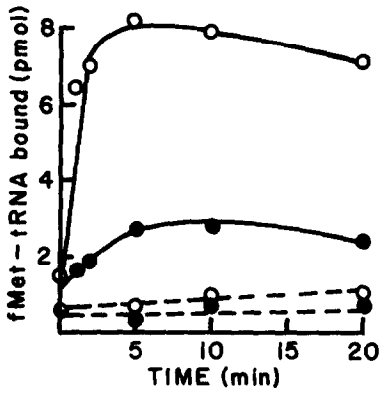

Fig.3. The effects of IF-3 and IF-1 on the rate of ribosomal dissociation. 70 S ribosomes (~18 pmol) were incubated at 25°C for the time periods indicated in the absence of IF-3 and IF-1 (–•––•–), with 3.0 μg IF-1 (–o––o–); with 1 5 μg IF-3 (–•–•–), and with 3.0 μg IF-1 and 1 5 μg IF-3 (–o–o–). The incubation mixture was then supplemented with IF-2, AUG, GTP and f[^{3}H]Met-tRNA, and the amount of f[^{3}H]Met-tRNA bound to ribosomes was determined as in section 2

bound was observed when the time of incubation was extended up to 20 min.

Reports from other laboratories [11,15] have indicated that the extent of dissociation obtained with IF-3 and the degree of stimulation obtained with IF-1 is greatly dependent upon the concentrations of monovalent and divalent cations. In all of the experiments described thus far, the concentrations of Mg^{2+} and NH_4^+ were 5 mM and 80 mM, respectively, in the first step of the assay. The data in fig.4 show the effects of altering the concentrations of Mg^{2+} and NH_4^+ in the first incubation mixture while maintaining these ions at 5 mM and 80 mM in the second incubation mixture. As seen in fig.4A lowering Mg^{2+} from 5 mM to 2.5 mM produced a slight increase in the amount of fMet-tRNA bound in the absence of IF-3 and IF-1, indicating that slightly more ribosomal dissociation was occurring at 2.5 mM Mg^{2+} in the absence of added factors. When the ribosomes were incubated at 2.5 mM Mg^{2+} with IF-3 alone, the amount of fMet-tRNA bound was considerably greater (about 4-fold) than that observed at 5 mM, and only a slight stimulation was obtained with IF-1. Raising Mg^{2+} to 7.5 mM decreased the amount of binding obtained with IF-3 alone and slightly increased the stimulatory

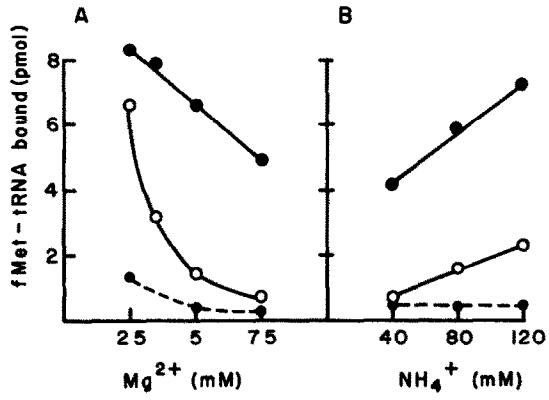

Fig 4. The effects of Mg^{2+} and NH_4^+ on the dissociation of 70 S ribosomes in the presence of IF-3 and IF-1 70 S ribosomes (~18 pmol) were incubated in the absence of IF-3 and IF-1 (–•––•–), in the presence of 1.5 μg IF-3 (–o–o–), and in the presence of 1 5 μg IF-3 and 3 0 μg IF-1 (–•–•–) In (A), the concentration of Mg^{2+} in the first incubation mixture was varied as indicated, the concentration of NH_4^+ was 80 mM In (B), the concentration of NH_4^+ in the first incubation mixture was varied as indicated, the concentrations of Mg^{2+} and NH_4^+ in the second incubation were adjusted to 5 mM and 80 mM, respectively.

effect of IF-1. As shown in fig.4B, raising the concentration of NH_4^+ produced effects similar to those obtained by lowering the concentration of Mg^{2+}.

4. Discussion

In this investigation we have developed a two-step assay procedure for measuring the dissociation of 70 S ribosomes. The procedure is especially useful in studying the effects of IF-1 and IF-3 on dissociation, since it confines the dissociation event to the first step and is therefore uncomplicated by any effect of IF-1 on the recycling of IF-2. Using this procedure, we find that IF-1 enhances both the rate and the extent of dissociation obtained with IF-3. The effect of IF-1 on the rate of dissociation can be readily explained on the basis of previous findings.

(i) IF-1 increases both the forward and backward rate constants for the reaction 70 S ⇌ 30 S + 50 S [12].

(ii) IF-1 increases the rate of subunit exchange [9].

If the sole function of IF-1 is to enhance the rate at which 30 S subunits become available for IF-3 to bind, then given sufficient time, the amount of dissociation obtained with IF-3 alone should approach that obtained with IF-1 and IF-3. However, we find that under certain experimental conditions (5 mM Mg^{2+}, 80 mM NH_4^+) the total amount of dissociation attainable with IF-3 alone remains 3–6-fold lower than that obtained with IF-1 and IF-3. These data indicate that IF-1 does more than enhance the rate at which 30 S subunits become available. Small increases in the extent of dissociation due to the addition of IF-1 have been reported by other investigators

We find, as have others [11,12,15], that the amount of ribosomal dissociation attainable with IF-3 alone is dependent upon the concentrations of Mg^{2+} and NH_4^+, and consequently, the degree of stimulation observed with IF-1 will vary, depending upon the concentrations of these ions. A possible explanation for these findings is that IF-1, Mg^{2+} and NH_4^+ affect the affinity of IF-3 for the 30 S subunit, thereby shifting the overall equilibrium further toward dissociation. A second possibility is that there are two populations of ribosomes, one which dissociates in the absence of IF-1 and one which does not dissociate in the absence of IF-1, and that the relative amounts of these two populations of ribosomes vary depending upon the ionic conditions, in particular the concentrations of Mg^{2+} and NH_4^+.

Recently it has been shown that after treatment of 70 S ribosomes with cloacin, which cleaves the 16 S RNA of the 30 S subunit, the ability of IF-1 to stimulate IF-3-mediated dissociation is lost [22]. In addition, it has been found that upon fixation with dimethyl suberimidate 30 S·IF-1 complexes, but not 70 S·IF-1 complexes, can be isolated [23]. These data indicate that IF-1 must exert its effect on the 30 S subunit. However, it is still unclear how the interaction between IF-1 and the 30 S subunit enhances both the rate and the extent of dissociation of 70 S ribosomes.

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