IF-3 REQUIREMENTS FOR INITIATION COMPLEX FORMATION WITH SYNTHETIC MESSENGERS IN *E. COLI* SYSTEM

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1. Introduction

The polypeptide chain initiation factor IF-3, which participates in the formation of the chain initiation complex, is generally believed to have a dual function:

1) It would ensure the availability of 30 S ribosomal subunits for initiation complex formation by promoting the dissociation of 70 S ribosomes [1,2]. This dissociation is usually ascribed to the binding of IF-3 to the 30 S sub-unit which would result in a shift of equilibrium 70 S $\rightleftharpoons$ 30 S + 50 S towards the right [3,4].

2) IF-3 would recognize some specific messenger starting signal on phage mRNA which is more complex than the initiator codon [5–7]. Consistent with this second function is the finding by the group of Bosch [8] that the binding of ribosomes to phage MS2-RNA requires IF-3, whereas binding to synthetic messengers (containing the initiator codon ApUpG and a random base sequence) can occur in the absence of this factor. The isolation of two messenger-discriminating species of IF-3 is also in favor of different classes of mRNA being recognized by IF-3 [9].

It is therefore critical to know whether IF-3 is required or not in the translation of synthetic mRNAs.

Several authors suggest that IF-3 is essential in the formation of the initiation complex, not only with phage mRNA, but also with synthetic messenger [10–15]. However, most of the experiments were performed with 70 S ribosomes, and the stimulating activity of IF-3 could be a consequence of its dissociating activity rather than an intrinsic activity in promoting the formation of the initiation complex, as might occur with phage mRNA. This interpretation is supported by the recent finding that IF-3 stimulates the binding of fMet-tRNA promoted by IF-2 + IF-1 in the presence of 70 S ribosomes, but not in the presence of 30 S + 50 S when they do not associate spontaneously, nor in the presence of 30 S alone [16].

The results reported here point out that with 30 S subunits, in the presence of synthetic mRNA, factor IF-2 alone is not sufficient to obtain maximum binding of fMet-tRNA, whatever its concentration; addition of either IF-3 or IF-1 markedly increases the binding at every IF-2 concentration. When both the 30 S + 50 S subunits are added, either separately or as 70 S couples, under conditions where dissociation of the ribosomes is not a limiting factor, IF-3 greatly enhances initiation complex formation in the presence of IF-2, but maximum binding occurs when all three factors are present. Furthermore, with the 30 S + 50 S, IF-3 promotes the recycling of IF-2, even in the absence of IF-1.

2. Materials and methods

2.1. *Crude extracts*

The crude initiation factors and high salt (1.5 M NH_4Cl) washed ribosomes were prepared (from *E. coli* MRE 600) as previously described [11].

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2.2. *Ribosomes*

The ribosomes were then purified on a 10–30% sucrose gradient in the following buffer: Tris–HCl (pH 7.5), 10 mM; NH_4Cl, 60 mM; Mg-acetate, 5 mM. The gradient was centrifuged 17 hr at 31 000 rpm in a Beckman T_{41} zonal rotor and the 70 S ribosomal fraction concentrated by further centrifugation 24 hr at 25 000 rpm in the rotor 30 (Spinco centrifuge). This purification yields ribosomes which are 80–90% associated at 5 mM Mg and are very active in protein synthesis [17].

The derived 30 S and 50 S ribosomal subunits were prepared by dissociation of the purified 70 S ribosomes and separated by zonal centrifugation (140

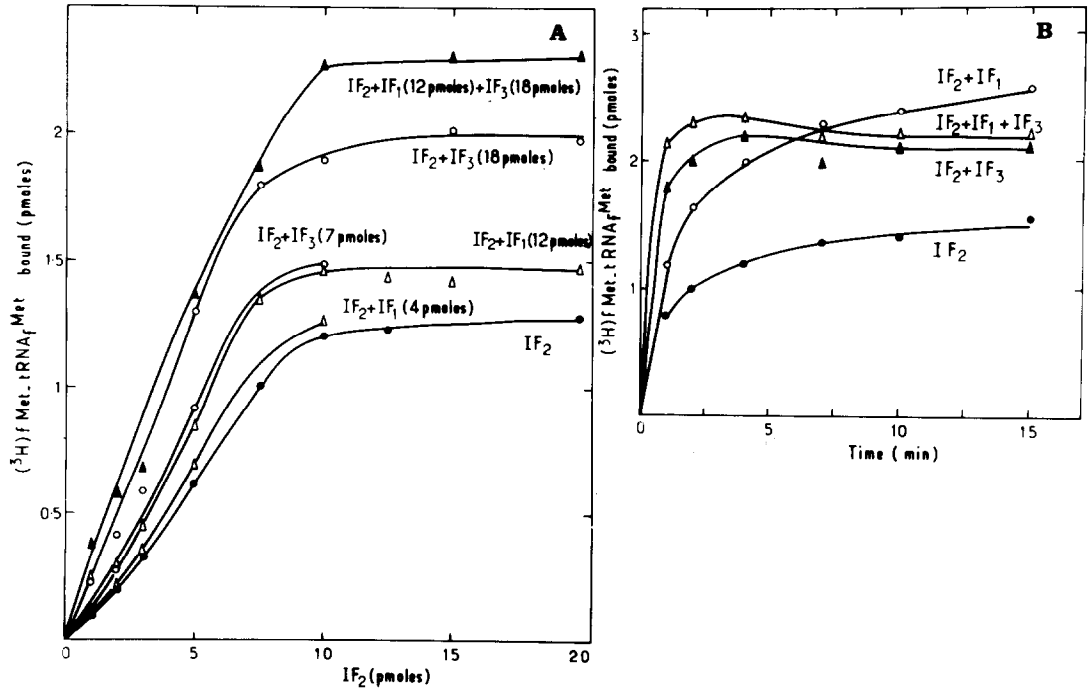

Fig. 1. Effect of IF-1 and IF-3 on fMet-tRNA binding with 30 S ribosomes: A. As a function of IF-2 concentration. The incubation mixture (100 μl) contained: Tris–HCl (pH 7.5), 50 mM; NH_4Cl, 50 mM; Mg-acetate, 5 mM; GTP, 1 mM, poly (A, U, G,) 0.15 A_{260} units; [^{3}H]fMet-tRNA, 18 pmoles (S.A. 2553 cpm/pmoles); IF-1, IF-2, IF-3, as indicated; 30 S ribosomes, 10.3 pmoles*. Incubation 15 min at 37°C. The binding effect was measured by the Millipore filtration technique; B. As a function of time. Same incubation mixture as above except that the amount of IF-1 was 12.5 pmoles; IF-2, 7.5 pmoles; IF-3, 14.5 pmoles; and the amount of 30 S, 9.3 pmoles. Incubation time (at 37°C) as indicated.

The concentration of initiation factors and ribosomes was calculated from UV. absorbance, using the following molar extinction coefficient:

$\epsilon^{1\text{ cm}}_{280\text{ nm}} = 1.0 \times 10^5$ for IF-2; $\epsilon^{1\text{ cm}}_{280\text{ nm}} = 1.0 \times 10^4$ for IF-1; $\epsilon^{1\text{ cm}}_{280\text{ nm}} = 6.7 \times 10^3$ for IF-3; $\epsilon^{1\text{ cm}}_{260\text{ nm}} = 1.4 \times 10^7$ for 30 S ribosomes; $\epsilon^{1\text{ cm}}_{260\text{ nm}} = 2.75 \times 10^7$ for 50 S ribosomes; $\epsilon^{1\text{ cm}}_{260\text{ nm}} = 4.15 \times 10^7$ for 70 S ribosomes.

mg per run) in the following buffer: Tris–HCl (pH 7.5), 10 mM; NH_4Cl, 60 mM; Mg-acetate, 10 mM; NaCl, 400 mM. The separated subunits were pelleted, resuspended in Tris–HCl (pH 7.5), 10 mM; Mg-acetate, 20 mM; NH_4Cl, 200 mM, and preincubated 15 min at 37°C in order to activate them before use.

2.3. *Initiation factors*

The mixture of crude initiation factors is dialyzed for 18 hr against 2 X 4 liters of: Tris–HCl (pH 7.4), 20 mM; NH_4Cl, 20 mM; glycerol 5%, and β-mercaptoethanol, 7 mM. The factors are then purified on a DEAE-cellulose column [11]. IF-1 is not adsorbed on the column, nor is a fraction of IF-3. The column is washed with: Tris–HCl (pH 7.4), 10 mM; NH_4Cl, 20 mM; and β-mercaptoethanol, 7 mM; some IF-3 is found in the wash. Elution on a 0.02–0.4 M NaCl gradient yields the remainder of IF-3 at 0.12 M and IF-2 at 0.23 M.

IF-2 is finally purified on a hydroxyapatite column as described be Lelong et al., and eluted at 0.17 M by a phosphate gradient 0.1 to 0.4 M (pH 7.4) [18].

The IF-1 + IF-3 collected from the void volume of the DEAE-cellulose column are adsorbed on a carboxymethyl-cellulose column (Whatman CM 52) and eluted by an ammonium chloride gradient (0.02–0.35 M) in 10 mM Tris–HCl (pH 7.5) containing 7 mM of β-mercaptoethanol. IF-1 and IF-3 elute respectively at 0.12 M and 0.25 M.

IF-1 is then adsorbed on a phosphocellulose column (Whatman P 11), according to Lee-Huang et al. [19] and eluted at 0.25 M by a 0.1–0.7 M gradient of NH_4Cl. IF-3 is purified as IF-1, but is eluted at 0.43 M [20]. Both IF-1 and IF-3 are homogeneous proteins, as shown by electrophoresis in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate.

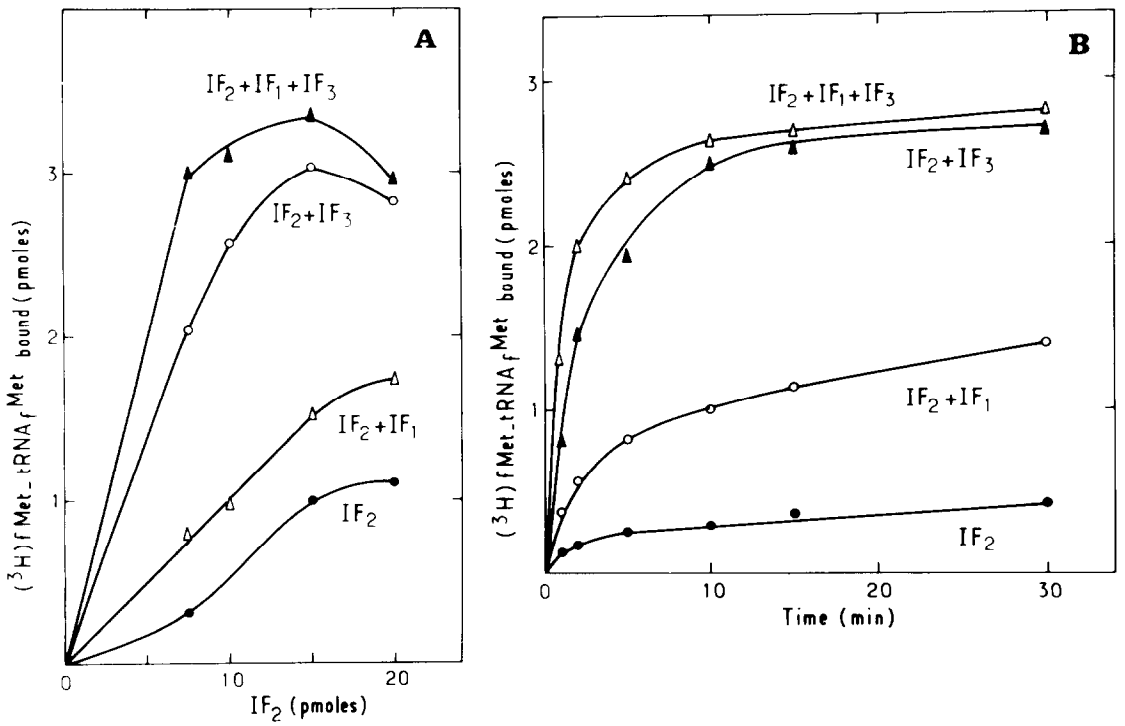

Fig. 2. Effect of IF-1 and IF-3 on fMet-tRNA binding with 30 S + 50 S ribosome: A. As a function of IF-2 concentration. Same conditions as for fig. 1B, except for the amount of ribosomes which were: 10.9 pmoles of 30 S + 11.3 pmoles of 50 S; B. As a function of time. Same conditions as for fig. 1B, except that the amount of ribosomes was: 9.3 pmoles of 30 S + 10.3 pmoles of 50 S.

2.4. fMet-tRNA was prepared according to Lelong et al. [18].

2.5. Poly (A, U, G) (base ratio, 1:1:1) was synthesized by polynucleotide phosphorylase and was a gift from Dr. Thang.

3. Results and discussion

In order to eliminate the possibility that, with synthetic mRNA, IF-3 functions purely as a dissociating factor, the requirement for its presence (as well as that of IF-1) was first tested with isolated 30 S subunits. Fig. 1A indicates the requirement for IF-3, IF-1, or both, as a function of IF-2 concentration, during prolonged incubations (15 min at 37°C). It can be seen that IF-3 (as well as IF-1) stimulates the fMet-tRNA binding at every concentration of IF-2. The stimulation by IF-3 is comparable to the one observed with IF-1. There is, however, a slight increase when both factors IF-3, IF-1 are added to IF-2, as compared to what is observed when they were added separately, but this effect is mostly seen at saturating amounts of IF-2.

Fig. 1B shows the effect of IF-3 and of IF-1 on the rate of fMet-tRNA binding, in the presence of IF-2; IF-3 increases this rate which is maximum in the presence of the three factors.

With 30 S + 50 S subunits, which under our experimental conditions are 50% associated (as determined by light scattering) [21,22] and where therefore the dissociation should not be a limiting factor, IF-3 drastically stimulates the binding of fMet-tRNA (whatever the mRNA concentration) at every concentration of IF-2, but the stimulation is higher at low concentration. (fig. 2A). Stimulation by IF-1 is much less than that by IF-3, in contrast to what was found with the 30 S subunits. When both factors are added to IF-2 the stimulation is higher than that observed with addition of IF-3 alone. This is particularly noticeable under conditions where the concentra-

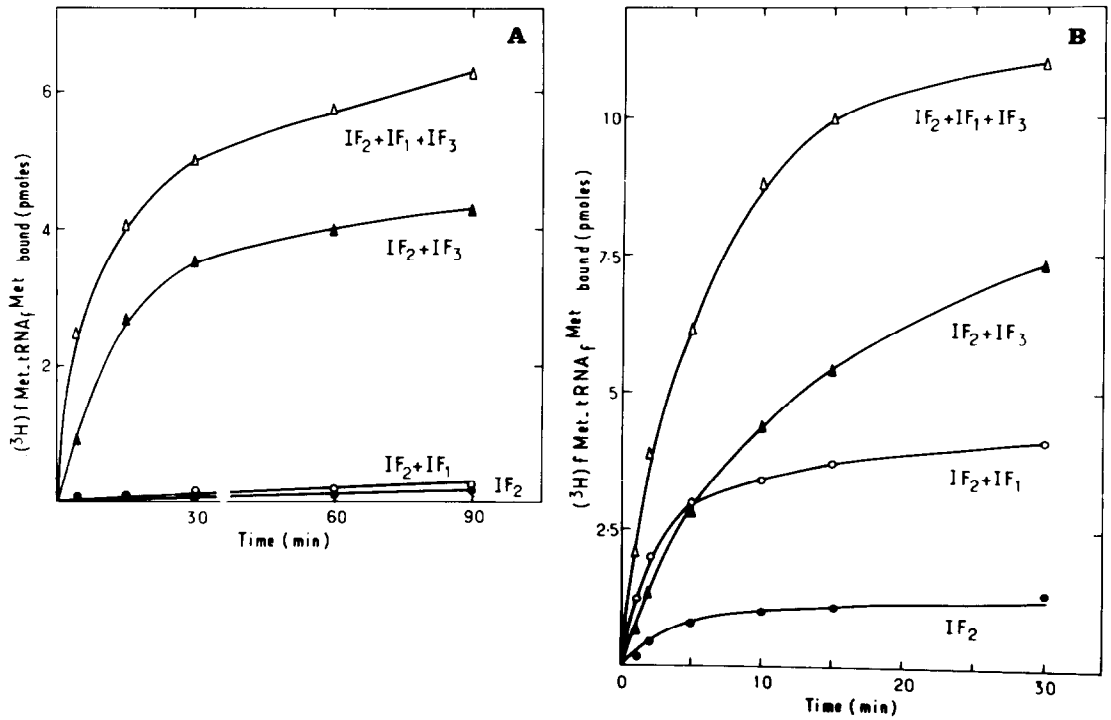

Fig. 3. Factor-dependence for the recycling of IF-2 with 30 S + 50 S ribosomes. Same incubation mixture as for fig. 1A exept for the amount of ribosomes: 39 pmoles of 30 S + 43 pmoles of 50 S; and factors: A. IF-2, 2.15 pmoles: IF-3, 18 pmoles; IF-1, 10.3 pmoles; B. IF-2, 7.5 pmoles; IF-3, 10.5 pmoles; IF-1, 14.5 pmoles. Incubation time (at 37°C) as indicated.

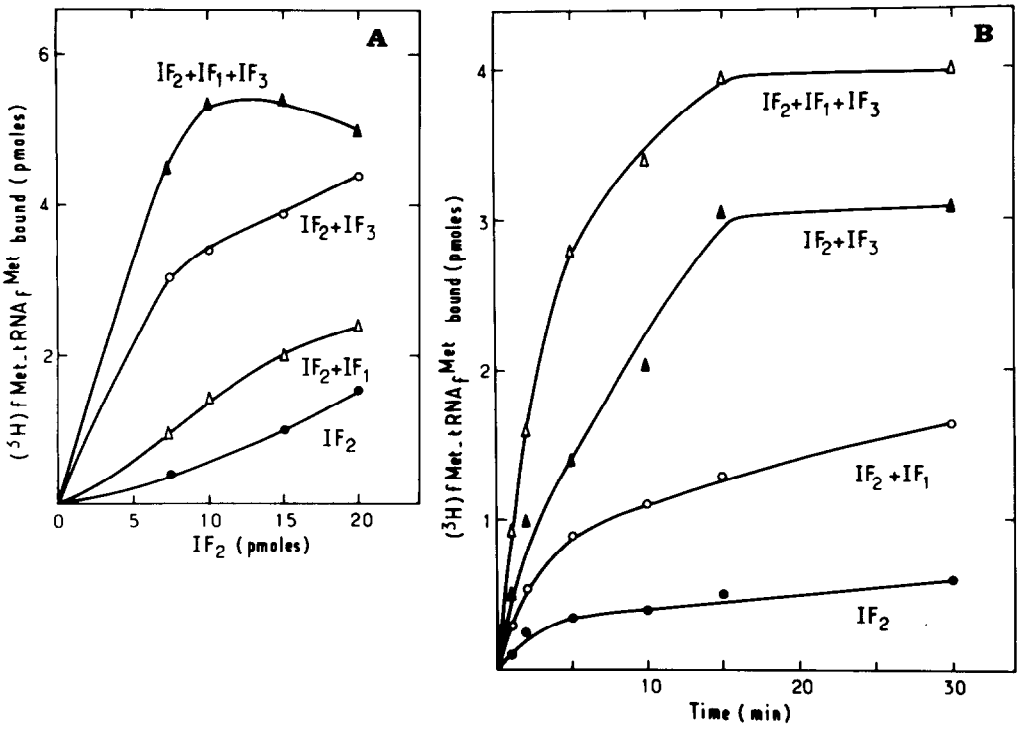

Fig. 4. Effect of IF-1 and IF-3 on fMet-tRNA binding with 70 S ribosomes: A. As a function of IF-2 concentration. Same conditions as for fig. 2A, except that the ribosomes were 70 S in the amount of 14.2 pmoles; B. As a function of time. Same conditions as for fig. 2B, except that the ribosomes were 70 S in the amount of 9.87 pmoles.

tion of ribosomes is not a limiting factor, with catalytic amounts of IF-2, conditions where IF-2 recycling should occur. This is shown in fig. 2B, where the ribosome concentration is a limiting factor, and fig. 3A and B where there is an excess of ribosomes and a limited amount of IF-2. It is clear, from fig. 3A that IF-3, in the absence of IF-1, is capable of stimulating the recycling of IF-2: 4 pmoles of fMet-tRNA are bound with 2 pmoles IF-2. One should also notice that in the presence of an excess of ribosomes and a minimal amount of IF-2, IF-1 does not promote fMet-tRNA binding appreciably, whereas IF-3 does.

Finally with 70 S ribosomes which are 80% associated, IF-3 is alone highly stimulatory, but addition of IF-1 to IF-2 + IF-3 markedly enhances the binding, even in the presence of stoichiometric amounts of IF-2, (that is non-recycling conditions) in contrast to what is observed with 30 S + 50 S. This is probably due to the strong stimulation, by IF-1, of the dissociating activity of IF-3 [21,22] and can clearly be seen when comparing fig. 2B and fig. 4B.

In conclusion the high stimulation by IF-3 of fMet-tRNA binding to isolated 30 S ribosomal subunits, as well as to 30 S + 50 S, under conditions where the dissociation is not limiting, indicates that IF-3 plays an active role in the formation of the 30 S initiation complex with synthetic mRNA; therefore its function is not limited to dissociating the ribosome. This does not eliminate the possibility of another role for IF-3 in the reaction which may involve messenger discrimination but this role remains to be rigorously demonstrated*.

* While this manuscript was being written a paper by Bernal et al., leading to a similar conclusion, appeared in the Proc. Natl. Acad. Sci. U.S., ref. [23].

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