RECYCLING OF THE INITIATION FACTOR IF-3 ON 30 S RIBOSOMAL SUBUNITS OF *E. COLI*

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1. Introduction

It has been proposed that during initiation of protein synthesis in *E. coli* the initiation factor IF-3 undergoes a cyclic process of association with the 30 S ribosomal subunit and dissociation therefrom at a certain stage of initiation complex formation [1]. Experimental support for such a model was presented by Sabol and Ochoa [2]. They found that adding ^{35}S-labeled IF-3 to a mixture of 70 S, 50 S and 30 S ribosomes resulted in IF-3 binding to the 30 S particles, but no radioactivity was found in the 70 S region. Furthermore they demonstrated that the factor was released as soon as a 70 S initiation complex, containing MS2 RNA and fMet-tRNA was formed. In basic agreement with these results were the data of Pon et al. [3] obtained with IF-3 labeled *in vitro* by reductive alkylation and those of Thibault et al. [4] with [^{35}S]IF-3.

Although these investigations suggested that IF-3 was released upon junction of the 50 S subunit to the 30 S initiation complex, the possibility could not be excluded that release occurred at an earlier stage. Kinetic investigations by Benne et al. [5] in which fMet-tRNA binding to 30 S ribosomes was studied with limiting amounts of IF-3 but non-limiting amounts of IF-2 suggested that IF-3 recycles at the level of the 30 S initiation complex. A similar suggestion is borne out by the experiment of fig. 1 in which f[^{3}H]Met-tRNA binding was followed in the presence of increasing amounts of IF-3. Virtually no difference in binding was observed whether or not the 30 S ribosomes were supplemented with 50 S particles even at limiting concentrations of IF-3.

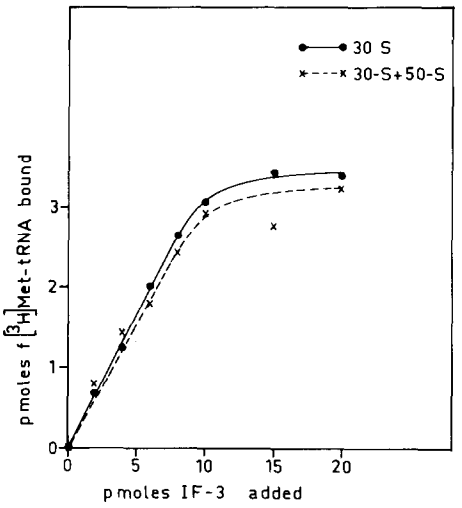

Fig. 1. IF-3 dependence of f[^{3}H]Met-tRNA binding to 30 S ribosomes in the absence and presence of 50 S particles. One hundred μg of 30 S ribosomes were incubated for 10 min at 37° in the presence of 4 μg of IF-2, 0.2 μg of IF-1, varying amounts of IF-3, 20 μg of MS2 RNA, 0.02 μmole GTP and 30 pmoles f[^{3}H]Met-tRNA in 0.1 ml of a buffer containing 7 mM Mg acetate, 50 mM K acetate, 50 mM Tris-HCl, pH 7.2, 10 mM NH_4Cl, 6 mM β-mercaptoethanol and 3% glycerol. The reaction was stopped by diluting the samples with 2 ml of buffer of the same ionic strength (buffer A) and filtering over Selectron BA 85/0. The filters were counted in toluene containing 0.7% PPO and 0.01% POPOP. Purified initiation factors IF-1 and IF-2 were prepared as described by Benne et al. [5] and IF-3 according to the slightly modified procedure of Sabol et al. [9]. (•–•–•) 50 S ribosomes absent; (X–X–X) 50 S ribosomes present.

2. Experimental and results

For a more direct approach ^{35}S-labeled IF-3 was prepared as described by Sabol and Ochoa [2] and purified by ammonium sulphate precipitation, phosphocellulose column chromatography and Sephadex G-100 gel filtration in 6 M urea. The purity of the factor was checked by SDS-gel electrophoresis on 10% polyacrylamide gels as described by Weber and Osborn [6] (compare fig. 2). The formation of initiation complexes was studied by sucrose gradient centrifugation and by equilibrium centrifugation on CsCl density gradients, respectively.

Upon association of the 30 S ribosomes with [^{3}H]-MS2 RNA a complex was formed which during sucrose gradient centrifugation sedimented somewhat faster than the single ribosomal particles. The complex was rather labile and dissociated during analysis. It was fixed therefore with 0.25% glutaraldehyde prior to centrifugation (compare legend of fig. 3). Under these conditions a small amount of ribosomal dimers was formed which usually displayed a slightly higher sedimentation rate than the 30 S–MS2 RNA complexes. The association between 30 S and the viral messenger requires the presence of IF-3 (compare figs. 3B and C), but IF-2 and IF-1 can be omitted (not illustrated). The latter two factors enhance the binding of MS2 RNA provided that IF-3 is present [7]. [^{35}S] IF-3 readily becomes bound to the 30 S particles (fig. 3A) and remains attached to the 30 S–MS2 RNA complex (fig. 3B), both in the presence and absence of initiation factors IF-1 and IF-2 [7]. However at the binding of f[^{3}H]Met-tRNA all [^{35}S] IF-3 is released from the complex (fig. 3D). The 30 S ribosomes which have not associated with MS2 RNA and sediment at the original 30 S position still contain IF-3. The fact that IF-3 is only released upon fMet-tRNA binding and the different extents of IF-3 binding to free 30 S ribosomes and 30 S–MS2 RNA complexes exclude artefactual binding due to fixation.

For a better separation of the 30 S–MS2 RNA complexes from non-bound 30 S ribosomes and MS2 RNA, CsCl-density gradient centrifugation was performed. An additional fixation with formaldehyde of the complexes was necessary in order to prevent disintegration during analysis (compare legend of fig. 4). The buoyant densities in a CsCl gradient of non-bound MS2 RNA and 30 S ribosomes are about 1.9 g/ml and

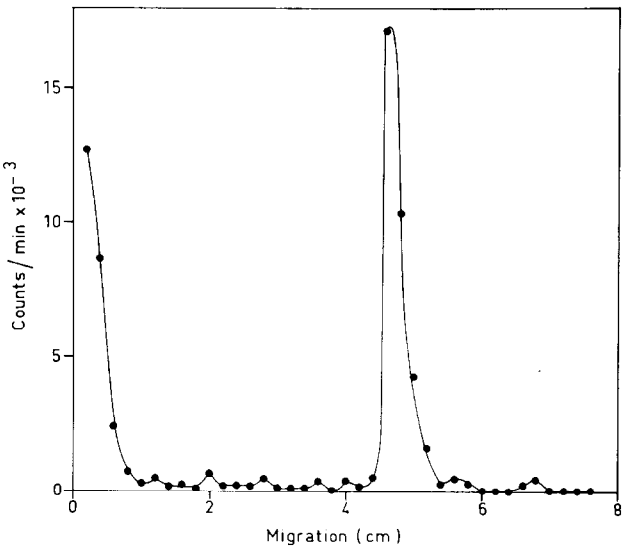

Fig. 2. Electrophoretic analysis of [^{35}S]IF-3 on polyacrylamide gel. [^{35}S]IF-3 (50,000 cpm) was submitted to electrophoresis on 8 cm gels of 10% acrylamide for 8 hr in 0.1% SDS (8 mA per gel). After electrophoresis the gel was cut in 1 mm slices which were incubated in 0.2 N KOH at 70° overnight. The radioactive samples were counted in Triton X-100 and toluene.

1.6 g/ml, respectively. They permit a good separation of these components from the 30 S–MS2 RNA complexes, the density of which is calculated to lie between 1.7 and 1.8 g/ml. As may be concluded from fig. 4B these complexes were formed to band at a density of 1.74 g/ml. The data of fig. 4A–D fully confirm the conclusions drawn from the sucrose gradient experiments (fig. 3). IF-3 is present in the 30 S–MS2 RNA complexes (fig. 4B), the formation of which is stimulated 3 to 4 times by IF-2 (not shown here). The ratio between IF-3 and MS2 RNA in the complexes is about 1:1. GTP does not affect the binding of IF-3 (not illustrated) and fMet-tRNA binding repels IF-3 from the complex (fig. 4D). Again no MS2 RNA binding to ribosomes in a system containing IF-1 and IF-2 but devoid of IF-3 coud be observed (fig. 4C). Artefactual binding due to fixation can be excluded on the same grounds as discussed above.

It is concluded that IF-3 recycles during initiation on the 30 S initiation complexes and is released therefrom before the junction of the 50 S subunit. As will be documented more extensively in a subsequent paper IF-2 is not a prerequisite from MS2 RNA bind-

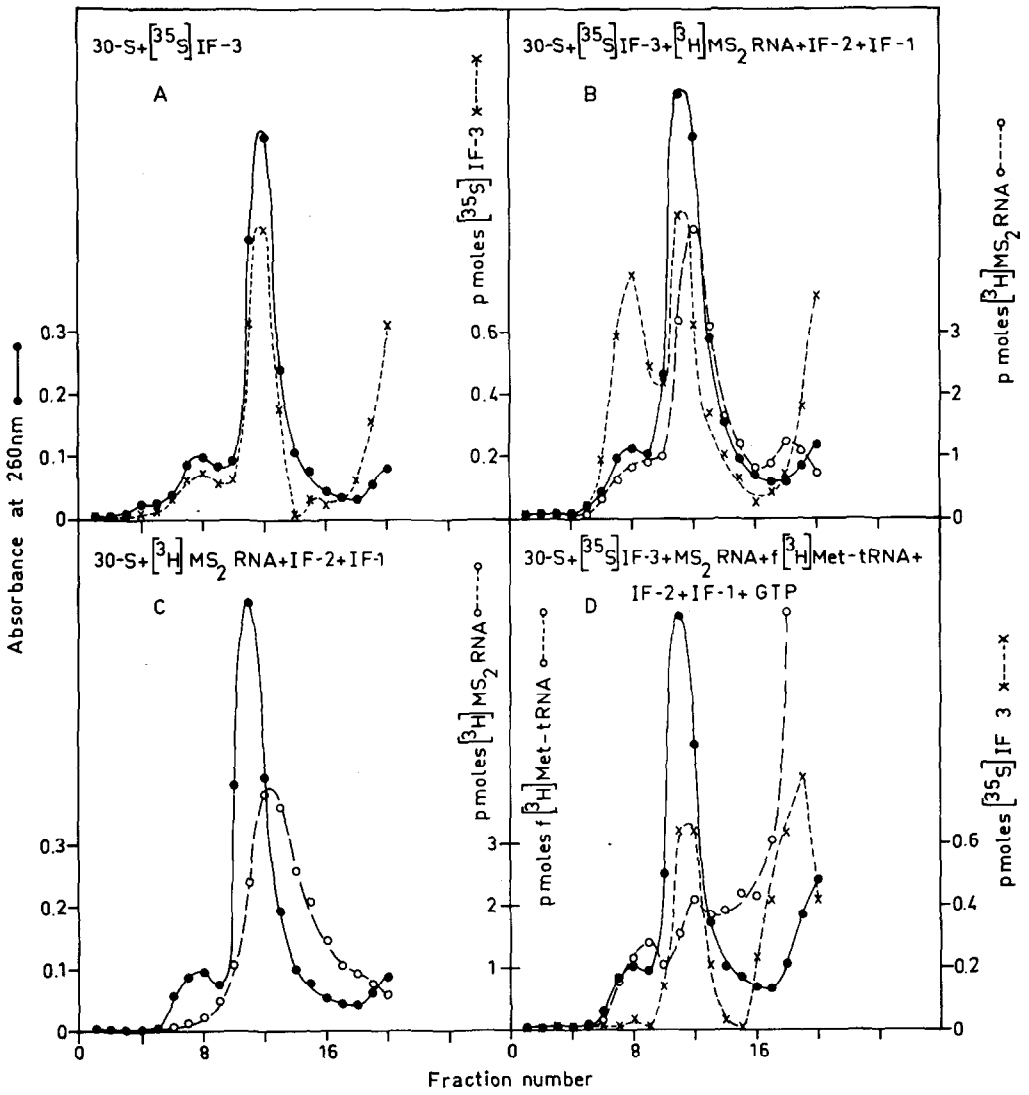

Fig. 3. Sucrose gradient analysis of [^{35}S]IF-3 binding to 30 S ribosomes in the presence and absence of [^{3}H]MS2 RNA and f[^{3}H]-Met-tRNA. Each reaction mixture (0.2 ml) contained 100 μg of 30 S ribosomes and 0.5 μg [^{35}S]IF-3 (specific radioactivity 5000 cpm per μg) in buffer A. When indicated in the figure the reaction mixtures were supplemented with 2 μg IF-2, 0.2 μg IF-1, 0.02 μmole GTP, 20 pmoles [^{3}H]MS2 RNA and 60 pmoles f[^{3}H]Met-tRNA. After incubation at 37° for 10 min, the mixtures were fixed by addition of 0.2 ml 0.5% glutaraldehyde in buffer A, layered on a sucrose gradient (10–30%) and centrifuged in a Spinco SW 27 rotor for 17 hr at 24,000 rpm. Optical density (260 nm) of each fraction (15 drops) was measured and radioactivity was counted in Triton X-100 and toluene.

ing to the 30 S particles, but the presence of IF-2 and IF-1 enhances this binding significantly (for a discussion of this problem see [8]).

Acknowledgement

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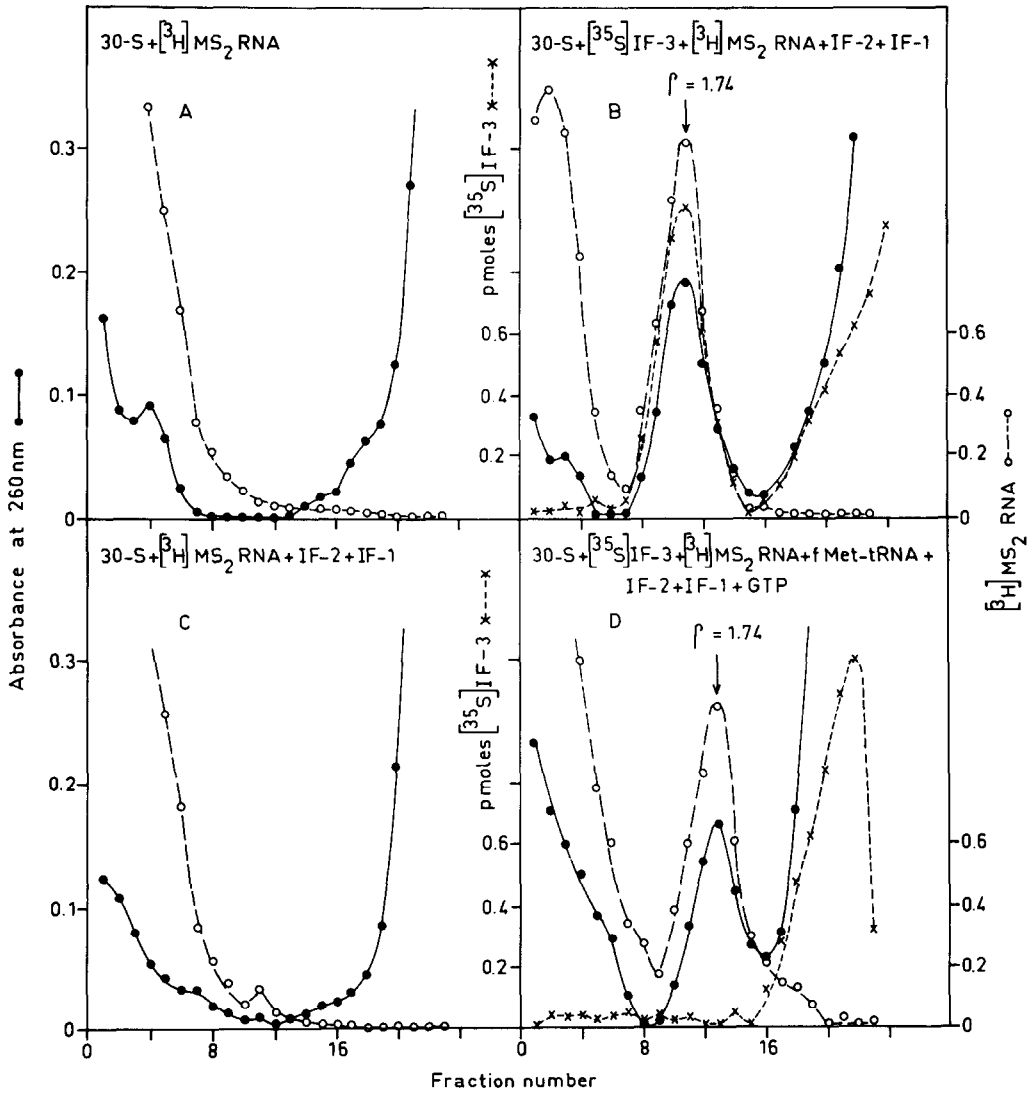

Fig. 4. Analysis of [^{35}S]IF-3 binding to 30 S ribosomes by means of equilibrium centrifugation in CsCl gradients. Binding was studied in the presence and absence of [^{3}H]MS2 RNA and fMet-tRNA as described in the legend of fig. 3. After incubation the reaction mixtures were diluted with 2 ml 0.25% glutaraldehyde in buffer A and fixed once more with 2 ml 10% formaldehyde in buffer A for 24 hr. Cesium chloride was added to a final density of 1.73 g/ml and Brij-58 to a final concentration of 1%. The samples were centrifuged at 25° in a Spinco SW 50-1 rotor for 48 hr at 30,000 rpm. Twenty three fractions were collected from each tube, diluted with 0.5 ml water and measured spectrophotometrically at 260 nm. The diluted fractions were then filtered over Selectron filters, which were subsequently dissolved in 1 ml ethylacetate. The final solutions were counted in 10 ml Triton X-100 and toluene.

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