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J. Biol. Chem. 1973, 248:4206-4212.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 248, No. 12, Issue of June 25, pp. 4206-4212, 1973
Printed in U.S.A.

The Interaction of Radioactive Initiation Factor IF-2 with Ribosomes during Initiation of Protein Synthesis*

(Received for publication, December 26, 1972)

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SUMMARY

The interaction of initiation factor IF-2 with ribosomes during initiation of protein synthesis was studied *in vitro* with the radioactive factor, [^{32}P]phosphoryl initiation factor IF-2. The binding and release of the factor were analyzed by sucrose density gradient centrifugation. The results show that:

1. Initiation factor IF-2 is part of the complete 30 S initiation complex formed in the presence of A-U-G, *N*-formylmethionyl transfer RNA, initiation factor IF-1, and 5′-guanosine triphosphate or 5′-guanylyl methylenediphosphonate.

2. Initiation factor IF-2 binds to 30 S ribosomes in the absence of the other components of the 30 S initiation complex. The binding is stabilized by the addition of 5′-guanosine triphosphate and by low salt concentrations in the gradients.

3. Initiation factors IF-1 and IF-3 greatly enhance the stability of initiation factor IF-2 binding to 30 S ribosomes. It is proposed that a complex of the 30 S ribosome and the three initiation factors may be an intermediate in the formation of the complete 30 S initiation complex.

4. Initiation factor IF-2 binds stoichiometrically to active 30 S ribosomes in the presence of saturating amounts of initiation factors IF-2, IF-1, and IF-3. If the binding reactions are treated with glutaraldehyde before gradient analysis, 2 molecules of initiation factor IF-2 are bound per 30 S ribosome.

5. Initiation factor IF-2 and *N*-formylmethionyl transfer RNA bind in equal proportions to the 70 S initiation complex formed with 5′-guanylyl methylenediphosphonate. When the 70 S complex is formed with 5′-guanosine triphosphate, however, initiation factor IF-2 no longer is bound to the complex. It is suggested that the role of 5′-guanosine triphosphate hydrolysis is to increase the rate of ejection of initiation factor IF-2 from the 70 S initiation complex.

Initiation factor IF-2 from *Escherichia coli* is clearly involved in at least two steps in the initiation of protein synthesis: the binding of fMet-tRNA to 30 S ribosomes (1), and the hydrolysis of GTP, which requires both the 30 S and 50 S ribosomal subunits (2–4). These two facts imply that IF-2 interacts with both the 30 S and 70 S ribosomes at specific times during the protein synthesis cycle. In the work reported here this problem has been studied directly with radioactively labeled IF-2.

Radioactive initiation factors have been used in several laboratories to study their binding to ribosomes. The labeled factor either can be isolated and purified from cells grown in a radioactive medium or it can be purified first and then labeled *in vitro* by attaching a radioactive chemical group which does not alter the factor's activity. Hershey *et al.* (5) isolated and purified [^{3}H]IF-1 from labeled cells several years ago using the first approach. The binding and release of IF-1 from 30 S ribosomes were studied by sucrose gradient centrifugation. It was shown that IF-1 is a component of the 30 S initiation complex, and that it is released from the ribosome when the 50 S joins the complex, before GTP hydrolysis has occurred.

More recently, [^{35}S]IF-3 was purified from labeled cells and its binding properties were studied (6). [^{35}S]IF-3 alone binds very tightly to 30 S ribosomes and is a part of the 30 S initiation complex. Like IF-1, it is released at the subunit junction step, before GTP hydrolysis. IF-3 also has been obtained radioactive by the second approach, *i.e.* by chemical modification. The pure factor was labeled with [^{14}C]methyl groups by reductive alkylation with [^{14}C]formaldehyde and sodium borohydride (7). The [^{14}C]CH_3-IF-3 exhibited essentially the same binding characteristics as did the [^{35}S]IF-3.

The isolation of IF-2 from small cultures grown in the presence of radioactive amino acids or sulfate has proven difficult, due to adsorption to glass surfaces of the small amounts of factor present. The recent finding in this laboratory (8) that pure IF-2 can be phosphorylated by rabbit skeletal muscle protein kinase has provided a simple method for labeling the factor. Since the ^{32}P-IF-2 thus prepared is fully active, it has been used in these studies to follow IF-2-ribosome interactions.

EXPERIMENTAL PROCEDURE

Materials—Biochemical compounds were obtained as follows: GTP from Calbiochem; guanylyl-5′-methylenediphosphonate from Miles Laboratories; dithiothreitol from Pierce Chemical Co., and glutaraldehyde 50%, biological grade, from Fisher Scientific. All other chemicals were reagent grade. A-U-G was prepared according to the method of Sundararajan and Thach

* This work was supported by grants from the American Cancer Society (NP-70), the United States Public Health Service (GM 17924), and Cancer Research Funds of the University of California.

(9). Sucrose, domestic grade, was purified by passage of a 72% w/v aqueous solution through a column of mixed bed resin, Amberlite AG 501-X8 (Bio-Rad). [^{3}H]fMet-tRNA was prepared from unfractionated *E. coli* B tRNA (Schwarz-Mann) and [^{3}H]-methionine (Schwarz-Mann, specific activity 2.8 Ci per mmole) according to the method of Hershey and Thach (10).

Growth of Cells—*E. coli* strain MRE600 was grown in 100-liter batches in a fermentor (New Brunswick model F-130) in a buffered, rich medium (11) (per liter: 20 g of glucose, 10 g of yeast extract (Difco), 34 g of KH_2PO_4, and 9 g of KOH; pH 6.8). Growth was carried out at 37° with vigorous stirring and aeration to mid-log phase (A_{550} = 5 in a Gilford 2400 S spectrophotometer), and was stopped by the addition of ice to the medium. The temperature was reduced to 0° over a period of 25 min. The cells were harvested by centrifugation (Sharples) and were washed once in 10 mM Tris-HCl, pH 7.5, and 10 mM Mg acetate. They were either used immediately or stored frozen at −70°.

Ribosomes—The preparation of ribosomes and their subunits is a modification of the procedures described by Noll (12). Cells (25 g) were broken by alumina grinding and unwashed ribosomes were prepared as described by Hershey *et al.* (13), except that the cell extraction buffer contained 10 mM Tris-HCl, pH 7.4; 10 mM Mg acetate; 100 mM NH_4Cl; and 1 mM dithiothreitol (Buffer A). The ribosomes (685 mg in 78 ml) were fractionated into 70 S ribosomes and native subunits in the Beckman Ti-15 zonal rotor by using an 8 to 25% hyperbolic sucrose gradient in Buffer A according to Eikenberry *et al.* (14). The fractions containing 70 S ribosomes free of native subunits were combined and concentrated by centrifugation (yield, 140 mg).

Ribosomal subunits were prepared from the 70 S ribosomes by sucrose gradient centrifugation in the Beckman Ti-14 zonal rotor. The purified 70 S ribosomes were diluted to 35 ml to a final concentration of 4 mg per ml in buffer containing 10 mM Tris-HCl, pH 7.5; 2 mM Mg acetate; 100 mM NH_4Cl; and 1 mM dithiothreitol (Buffer B). Directly following the dilution, the ribosome sample was loaded onto a 390-ml 10 to 30% hyperbolic sucrose gradient in Buffer B. The contents were centrifuged at 47,000 rpm for 3½ hours and then collected in fractions of 10 ml. Each fraction was immediately brought to 10 mM in Mg acetate. The fractions containing pure 30 S and pure 50 S ribosomes were combined. The ribosomal subunits were pelleted by centrifugation at 40,000 rpm for 10 hours in the Beckman Ti-60 rotor and were suspended in Buffer A and stored in small aliquots at −70° at about 30 mg per ml.

Yields of 27 mg of 30 S ribosomes and 67 mg of 50 S ribosomes were obtained. The 30 S ribosomes were pure by analysis by sucrose gradient centrifugation in Buffer A. The 50 S ribosomes were slightly contaminated (less than 5%) by 70 S ribosomes. The two subunits, when mixed in equimolar amounts, showed greater than 90% reassociation to 70 S ribosomes in Buffer A. The 30 S ribosomes were very active: 1 equivalent of 30 S ribosomes bound 0.69 equivalents of fMet-tRNA in the presence of A-U-G, GTP, and saturating amounts of IF-1 and IF-2, as measured by filtration through Millipore HAWG filters (13). Heating (15) did not result in increased activity. Although the subunits were never exposed to salt concentrations greater than 100 mM, they were completely dependent on the addition of all three initiation factors in the fMet-tRNA binding assay with R-17 RNA.

Initiation Factors—Initiation factors were isolated and purified from *E. coli* strain MRE600, grown as described above. IF-1 was prepared by the method of Hershey *et al.* (5). IF-3 was purified by ammonium sulfate fractionation (55 to 70% saturation) of a 0.75 M NH_4Cl ribosomal wash, and salt gradient elution from a phosphocellulose column.[1] IF-2 was purified by ammonium sulfate fraction (30 to 50% saturation) of a 0.5 M NH_4Cl ribosomal wash, and salt gradient elution from phosphocellulose and DEAE-Sephadex columns.[2] Each factor was greater than 90% pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

^{32}P-IF-2 was prepared by phosphorylation of pure IF-2 with rabbit muscle protein kinase, as described by Fakunding *et al.* (8). Of the two forms of IF-2 purified and phosphorylated, only the larger form, ^{32}P-IF-2a (mol wt 91,000),[3] has been used in these studies. The specific activity of the ^{32}P-IF-2, calculated from measurements of radioactivity, protein concentration (determined by the method of Lowry *et al.* (16)), and molecular weight, is given in the Figure legends

Sucrose Gradient Analyses—The composition of the gradients and the conditions of centrifugation are described in the figure legends. The location of ribosomes was determined by passing the gradients through a flow cell (2-mm light path) and recording absorbance at 260 nm with a Gilford 2400 S spectrophotometer. Fractions of about 0.15 ml for 4.6-ml gradients, and 0.55 ml for 13-ml gradients, were collected inton scitillation vials. Water was added to a final volume of 0.6 ml and 5 ml of scintillation fluid (2 parts toluene containing per liter 6 g of 2,5-diphenyloxazole (PPO) and 0.075 g of 1,4-bis[2-(5-phenyloxazole)]benzene (POPOP); 1 part Triton X-100) were added. The radioactivity was counted in a Beckman LS-200 scintillation counter; tritium was counted with 19% efficiency, and was corrected for 10% crossover of ^{32}P counts.

RESULTS

IF-2 Binds to 30 S Ribosomes—The binding of ^{32}P-IF-2 to the 30 S initiation complex in the presence of [^{3}H]fMet-tRNA, A-U-G, IF-1, and GTP can be demonstrated by sucrose gradient centrifugation (Fig. 1A). This result shows directly that IF-2 is a constituent of the complete initiation complex. It is consistent with earlier results which demonstrated that the binding of fMet-tRNA to 30 S ribosomes is proportional to the amount of IF-2 added, when saturating amounts of IF-1, mRNA, and GTP are used (17). The binding of ^{32}P-IF-2 and [^{3}H]fMet-tRNA to the 30 S complex occurs to the same extent when Gpp(CH$_2$)p[4] replaces GTP (Fig. 1B). The profiles are not altered appreciably if GTP or Gpp(CH$_2$)p are omitted in the analytical gradients.

^{32}P-IF-2 alone will bind to 30 S ribosomes (Fig. 2, *open circles*). The presence of the other components in the complete initiation complex are not necessarily required for the binding reaction (see below). Such binding is observed only with 30 S ribosomes; no interaction with 50 S or vacant 70 S ribosomes (free of bound mRNA and tRNAs) is observed under these conditions (results not shown).

Stability of IF-2 Binding—The stability of the IF-2-30 S complex formed in the absence of the other components is strongly dependent on the monovalent cation concentration in the analytical gradient. Although the complex is formed in a

[1] K. Johnston and J. W. B. Hershey, unpublished results.

[2] J. L. Fakunding, K. Johnston, and J. W. B. Hershey, manuscript in preparation.

[3] In order to be in agreement with the initiation factor nomenclature of others in the field, we have reversed the names of the two forms of IF-2 previously described (8), and now call the larger protein IF-2a and the smaller, IF-2b.

[4] The abbreviation used is: Gpp(CH$_2$)p, guanylyl-5′-methylenediphosphonate.

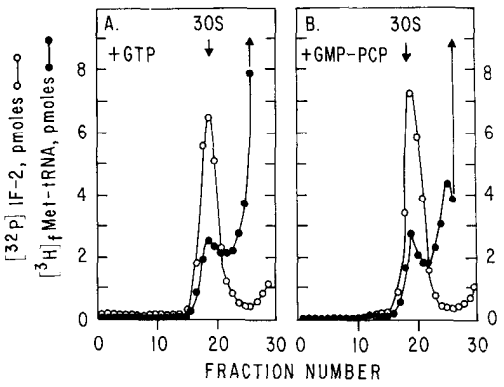

FIG. 1. Formation of 30 S initiation complex with GTP or Gpp(CH$_2$)p (*GMP-PCP*). Reaction mixtures (0.1 ml) contained 10 mM Tris-HCl, pH 7.4; 100 mM NH_4Cl; 5 mM Mg acetate; 1 mM dithiothreitol; 60 pmoles (0.83 A_{260} units) of 30 S ribosomes; 0.2 mM GTP (*Panel A*) or Gpp(CH$_2$)p (*Panel B*); 25 μM A-U-G; 40 pmoles of [^{3}H]fMet-tRNA (specific activity 2.8 Ci per mmole); 0.4 μg of IF-1; and 4 μg of ^{32}P-IF-2 (320 cpm per pmole). The mixtures were incubated for 10 min at 30°, chilled and layered onto 4.6-ml linear 7 to 25% sucrose gradients containing 10 mM Tris-HCl, pH 7.4; 40 mM NH_4Cl; 5 mM Mg acetate; 1 mM dithiothreitol; and 0.04 mM GTP (*Panel A*) or Gpp(CH$_2$)p (*Panel B*). The gradients were centrifuged in a Beckman SW 56 rotor at 40,000 rpm for 160 min at 4°

reaction mixture containing 100 mM salt, recovery of the complex is accomplished only in a gradient of low ionic strength (Fig. 2, *open circles*). Thus, while IF-2 binding to 30 S ribosomes is quite stable in gradients containing 20 mM NH_4Cl (Fig. 2A), the binding is labilized at higher salt concentrations (Fig. 2, *B* and *C*) to the point where little or no binding is detectable at 60 mM salt. Increasing the ionic strength in the gradient tends to shift the IF-2-30 S interaction toward dissociation. The stability of factor binding to ribosomes during gradient centrifugation is discussed in detail below.

The addition of GTP, both to the binding reactions and to the gradients, stabilizes the binding (Fig. 2, *closed circles*). Inspection of the gradient profiles reveals that the presence of GTP confers on the system about the same amount of stabilization as lowering of the salt concentration by 10 or 20 mM. Thus, the presence of GTP shifts the equilibrium toward association of the components. This effect of GTP is not seen with Gpp(CH$_2$)p or GDP however. In the presence of either of the latter two nucleotides, the stabilities of the IF-2-30 S complex resemble those obtained in the absence of GTP.

Factors IF-1 and IF-3 also stabilize the binding of IF-2. IF-2 alone interacts weakly with the 30 S ribosome as mentioned above. A large part of the bound IF-2 apparently leaves the 30 S particle during centrifugation (Fig. 3A). If either IF-1 or IF-3 is added with the ^{32}P-IF-2, only a slight effect on the binding is observed (Fig. 3, *B* and *C*). However, in the presence of both IF-1 and IF-3, stable binding is realized and the IF-2 co-sediments with the 30 S peak (Fig. 3*D*). It appears that the three factors behave in a cooperative manner to form a stable factor-30 S ribosomal complex, even in the absence of fMet-tRNA, mRNA, and GTP. It is not yet known whether the presence of GTP confers even more stability to this complex. While the presence of all three factors in the complex is not directly demonstrated here, it has been shown previously that IF-1 and IF-3 do bind to and co-sediment with the 30-S ribosome under these conditions (5–7).

The stability of the IF-2-30 S complex is somewhat variable

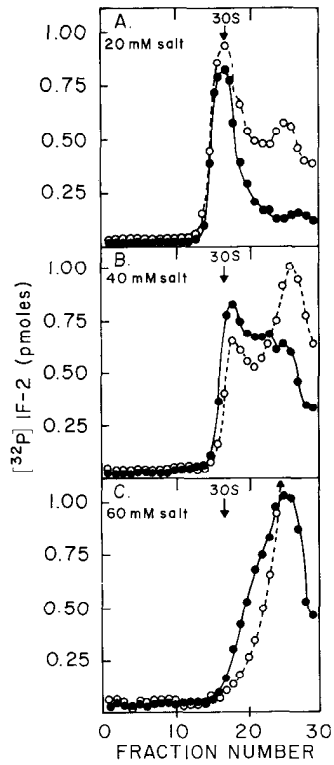

FIG. 2. Effect of GTP and salt concentration on IF-2 binding to 30 S ribosomes. Reaction mixtures (0.1 ml) contained 50 mM Tris-HCl, pH 7.4; 100 mM NH_4Cl; 5 mM Mg acetate; 7 mM β-mercaptoethanol; 0.2 mM GTP (●); 60 pmoles of 30 S ribosomes; and 22 pmoles of ^{32}P-IF-2 (1800 cpm per pmole). They were incubated for 10 min at 30°, chilled, and layered onto 4.6-ml linear 7 to 25% sucrose gradients containing 50 mM Tris-HCl, pH 7.4; 5 mM Mg acetate; 7 mM β-mercaptoethanol; NH_4Cl as indicated in the *panels*; and 0.04 mM GTP (●). The gradients were centrifuged at 40,000 rpm for 180 min at 4° in a Beckman SW 56 rotor.

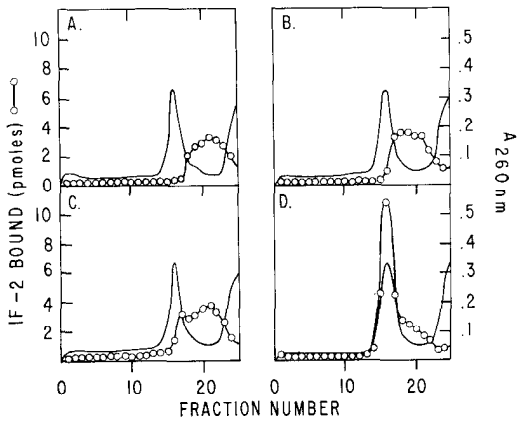

FIG. 3. Effect of IF-1 and IF-3 on IF-2 binding to 30 S ribosomes. Reaction mixtures (0.1 ml) contained 10 mM Tris-HCl, pH 7.4; 100 mM NH_4Cl; 6 mM Mg acetate; 1 mM dithiothreitol; 1 mM GTP; 125 pmoles of 30 S ribosomes; 66 pmoles of ^{32}P-IF-2 (100 cpm per pmole); and, where indicated, 75 pmoles of IF-1 and 80 pmoles of IF-3. The mixtures were incubated for 5 min at 30°, diluted with 0.3 ml of buffer (10 mM Tris-HCl, pH 7.4; 10 mM Mg acetate; and 7 mM β-mercaptoethanol), and layered onto 13-ml linear 7 to 20% sucrose gradients containing 10 mM Tris-HCl, pH 7.4; 10 mM Mg acetate; 20 mM NH_4Cl; and 7 mM β-mercaptoethanol. The gradients were centrifuged in a Beckman SW 40 rotor at 39,000 rpm for 180 min at 4°.

with respect to the batch of 30 S ribosomes employed. The ribosomes used in Fig. 3 bound IF-2 alone poorly when analyzed in 20 mM salt gradients, while those used in Fig. 2 bound IF-2 very well under similar conditions. Only when analyzed in 60 mM salt gradients did the ribosomes of Fig. 2 show a weak interaction. Other ribosome preparations, all prepared by the procedure described under "Experimental Procedure," showed binding characteristics between these two extremes. The possibility that the variable stability of IF-2 binding can be correlated with the presence or absence of specific 30 S ribosomal proteins is currently under investigation.

Stoichiometry of Binding—Increasing amounts of ^{32}P-IF-2 were incubated with a constant amount of 30 S ribosomal subunits in the presence of excess IF-1 and IF-3. The binding mixtures were analyzed by centrifugation in sucrose gradients containing sufficient NH_4Cl (40 mM for the ribosomes used here) to labilize any IF-2 binding without IF-1 and IF-3. Thus the IF-2 bound is only that which is specifically stabilized by the presence of IF-1 and IF-3. The picomoles of IF-2 actually recovered in the 30 S region of the gradients were plotted against the picomoles of IF-2 added to the incubation mixtures (Fig. 4, *closed circles*). If it is assumed that each particle binds one IF-2 molecule, the plateau value of IF-2 binding indicates that 70% of the 30 S ribosomal subunits are active. The ability of the 30 S ribosomes to bind IF-2 correlates well with their activity in fMet-tRNA binding, as measured by the Millipore filtration assay (13); at saturating IF-2 concentrations, 0.69 equivalents of fMet tRNA were bound per 30 S particle.

Another approach for determining the stoichiometry of IF-2 binding to 30 S ribosomes is first to treat the IF-2-30 S complex with glutaraldehyde and then to analyze the stably fixed complex by sucrose gradient centrifugation. As shown in Fig. 4, the results of glutaraldehyde fixation of IF-2 bound to 30 S ribosomes with IF-1 and IF-3 (*open circles*) and IF-2 bound to 30 S alone (*open triangles*) are similar to each other, but are in striking contrast to those obtained without glutaraldehyde treatment. The binding at low IF-2 concentrations is nearly linear up to approximately 0.7 IF-2 bound per ribosome. At higher IF-2 concentrations, IF-2 binding begins to approach a plateau value where nearly 2 moles of IF-2 are bound per mole of 30 S particle. This second mode of binding is seen only when glutaraldehyde fixation is used. It indicates either a second site of weaker IF-2 binding or a nonspecific interaction. The second site is obviously not that occupied by IF-1 or IF-3.

The initial slope of both kinds of IF-2 saturation curves in Fig. 4 is approximately 0.5, which might be interpreted to mean that only one-half of the IF-2 molecules are active. A more likely explanation however is the following. Only about 50% of the ^{32}P-IF-2 added to the incubation reactions is actually recovered from the gradients. The loss of ^{32}P radioactivity seems to occur primarily during incubation of the binding reaction, and is probably due to adsorption of the ^{32}P-IF-2 to the surface of the incubation tube. Thus only 50 to 60% of the original ^{32}P is applied to the gradients, and the subsequent recovery of the material actually centrifuged is greater than 80% (30 S recovery is essentially 100%). Therefore, if the abscissa of Fig. 4 were altered so that the values were those of ^{32}P-IF-2 added to the gradient instead of added to the incubation, the initial slope would approximate 1.0 instead of 0.5. The ^{32}P-IF-2 preparation would then appear to be essentially totally active, which is consistent with the ^{32}P-IF-2 profile in Fig. 2*A*, where greater than 80% of the recovered IF-2 is found in the 30 S region of the gradient.

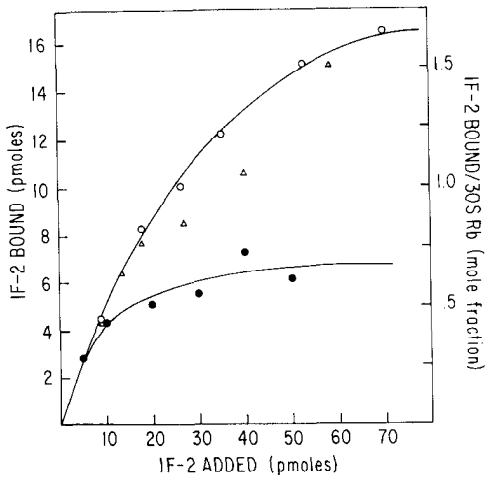

FIG. 4. Saturation binding of ^{32}P-IF-2 to 30 S ribosomes. Reaction mixtures (0.1 ml) contained 10 mM Tris-HCl, pH 7.4; 100 mM KCl; 5 mM Mg acetate; 1 mM dithiothreitol; 2 mM potassium phosphate, pH 7.4; 10 pmoles of 30 S ribosomes; 75 pmoles of IF-1 and 80 pmoles of IF-3 when indicated; and ^{32}P-IF-2 as shown. The mixtures were incubated for 10 min at 30°, chilled, and either layered immediately onto gradients or treated with glutaraldehyde as follows. To each mixture was added first 30 μg of bovine serum albumin and then enough glutaraldehyde to reach a final concentration of 2.5% w/v. Untreated mixtures were analyzed in 4.6-ml linear 7 to 25% sucrose gradients containing 10 mM Tris-HCl, pH 7.4; 40 mM NH_4Cl; 5 mM Mg acetate; and 1 mM dithiothreitol. The gradients were centrifuged in a Beckman SW 56 rotor at 40,000 rpm for 200 min at 4°. Glutaraldehyde-treated binding mixtures were analyzed in gradients of the same composition except that 50 mM KCl replaced 40 mM NH_4Cl. The gradients were centrifuged in a Beckman SW 56 rotor at 50,000 rpm for 120 min at 4°. The picomoles of bound ^{32}P-IF-2 were calculated from the sum of the ^{32}P counts in the 30 S region of the gradient. ●——●, with IF-1 and IF-3, without glutaraldehyde treatment; ○——○, with IF-1 and IF-3, with glutaraldehyde treatment; △——△, without IF-1 and IF-3, with glutaraldehyde treatment.

IF-2 Binds to 70 S Ribosomes—In order to determine the fate of IF-2 at later stages of the initiation process, 70 S initiation complexes were studied. Complete 30 S initiation complexes were formed with GTP or Gpp(CH_2)p as described in Fig. 1, and 50 S ribosomes were then added. The binding of ^{32}P-IF-2 and [^{3}H]fMet-tRNA to the ribosomes was analyzed by centrifugation in sucrose gradients, and the results are shown in Fig. 5.

In the presence of Gpp(CH_2)p, both IF-2 and fMet-tRNA were found predominantly in the 70 S region in nearly equimolar amounts (Fig. 5*A*). The amounts of [^{3}H]fMet-tRNA and ^{32}P-IF-2 found in the 70 S region were proportional to the amount of ^{32}P-IF-2 added to the incubation reactions. The results clearly demonstrate that IF-2 and fMet-tRNA bind in equal proportions to 70 S ribosomes in the presence of Gpp(CH_2)p.

The Gpp(CH_2)p-mediated binding of IF-2 to 70 S ribosomes depends on the presence of fMet-tRNA and A-U-G; when the assay contained ^{32}P-IF-2, Gpp(CH_2)p and 70 S ribosomes, but not the other components of the initiation complex, the IF-2 bound only to contaminating 30 S ribosomes. Binding also depended on low salt in the sucrose gradients; concentrations of NH_4Cl above 40 mM resulted in poor IF-2 binding to the 70 S complex. The presence of IF-1 was not required, however, and apparently did not alter the stability of the 70 S complex. Stable IF-2 binding depended specifically on the presence of Gpp(CH_2)p, not only in the incubation reaction but in the gradient as well.

IF-2 Is Released from 70 S Ribosomes after GTP Hydrolysis—When the 70 S complex formed with GTP was analyzed, the

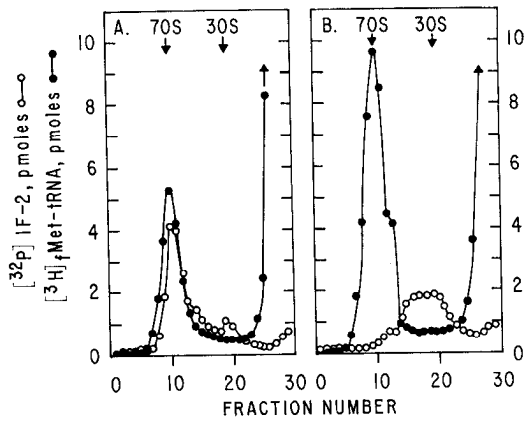

FIG. 5. The interaction of ^{32}P-IF-2 with the 70 S initiation complex. Complete 30 S initiation complexes were formed with Gpp(CH$_2$)p or GTP, as described in Fig. 1. After the 10-min incubation at 30°, 66 pmoles of 50 S ribosomes were added and the incubation was continued for an additional 5 min. The mixtures were chilled and analyzed by sucrose gradient centrifugation as described in Fig. 1. *A*, with Gpp(CH$_2$)p; *B*, with GTP.

results differed significantly from those described above with Gpp(CH$_2$)p. As seen in Fig. 5*B*, very little ^{32}P-IF-2 was found in the 70 S region of the gradient. The reduced stability of the IF-2-70 S complex is most likely due to hydrolysis of the GTP in the complex. That a weak interaction still exists between IF-2 and the fMet-tRNA-70 S complex is indicated by the broad profile of ^{32}P-IF-2 in the 50 S region of the gradient. The IF-2 in this region of the gradient must originate from interaction with the 70 S complex and not with the 50 S ribosomes, since as mentioned earlier, no stable binding of ^{32}P-IF-2 to pure 50 S ribosomes was seen.

The profiles shown in Fig. 5*B* (with GTP) show that [^{3}H]fMet-tRNA is bound to 70 S ribosomes in amounts substantially greater than those seen in Fig. 5*A* (with Gpp(CH$_2$)p). This difference between the amount of fMet-tRNA bound in the presence of GTP or Gpp(CH$_2$)p is greatest with low (limiting) concentrations of IF-2. The increased fMet-tRNA binding in the presence of GTP implies that IF-2 acts catalytically with the result that 1 molecule of IF-2 can participate in the formation of several initiation complexes. This catalytic activity of IF-2 is discussed in greater detail below.

When all guanosine nucleotides were omitted from the reactions leading to the formation of the 70 S initiation complex, or when GDP was added instead, the ^{32}P-IF-2 and [^{3}H]fMet-tRNA profiles were altered. In both cases the ^{32}P-IF-2 profile resembled the one obtained in the presence of GTP (Fig. 5*B*), while the [^{3}H]fMet-tRNA profile was similar to the one obtained in the presence of Gpp(CH$_2$)p (Fig. 5*A*).

DISCUSSION

Initiation factor IF-2 binds to 30 S but not to 50 S ribosomes. The results show by direct measurement that IF-2 is an integral part of the 30 S initiation complex. The other components of the complex have already been identified: fMet-tRNA (18), A-U-G (19) or mRNA (20, 21), GTP (22), IF-1 (5), and IF-3 (6, 7).

The stabilities of binding of each of the three initiation factors with 30 S ribosomes are not identical. The IF-2 interaction appears to be more stable than IF-1 binding (5) but less stable than IF-3 binding (6, 7). Since the stability of the IF-2-30 S complex is dependent upon the particular ribosomal preparation used, comparison of the binding stabilities of the three factors should be carried out with the same preparation of 30 S ribosomes. Such experiments are currently being done in this laboratory.

The implications of the results on the stability of factor binding, as determined by sucrose gradient centrifugation, to the mechanism of the initiation process are not obvious. ^{32}P-IF-2 was found not only at the top and in the 30 S region of the gradients, but also in the region through which the 30 S complex had sedimented. This indicates that during centrifugation the IF-2-30 S complex either dissociated irreversibly, or that it was in dynamic equilibrium with free IF-2 and 30 S ribosomes. In the former case, stability of binding would depend simply on the rate of dissociation of the complex. In the latter case, the rates of both the factor binding and the complex dissociation reactions would be involved, and variations in stability could reflect a change in either one alone or in both. Thus the effects of GTP, low salt concentrations, or the presence of IF-1 and IF-3 could be to decrease the rate of IF-2-30 S ribosome dissociation, or to increase the rate of IF-2 binding to the ribosomes. From studies of the kinetics of fMet-tRNA binding to 30 S ribosomes,[5] it can be assumed that the rate of IF-2 binding is very fast (essentially complete in less than 5 s under the conditions employed in these studies), since IF-2 is required to bind fMet-tRNA. Since no precise measurement of the rate of the IF-2-30 S dissociation reaction has been made, however, we cannot determine which rate constants are affected by those compounds conferring binding stability. Sucrose gradient centrifugation is relatively slow and may not be a suitable method for determining the rates of factor-ribosome interactions.

The requirement for all three initiation factors in order to obtain the most stable binding of IF-2 to 30 S ribosomes suggests that the three factors bind cooperatively. The obligatory presence of IF-1 and IF-3 for this stabilization provides another measure of activity for these factors. The results also suggest that the factors may be contiguously situated on the ribosomal surface. The isolation of an IF-2-IF-3 complex by Groner and Revel (23) supports this hypothesis.

The fact that IF-2, and in addition IF-1 and IF-3, can bind to the 30 S ribosome suggests that such complexes may be intermediates in the formation of the 30 S initiation complex. The sequential interaction of initiation factors with 30 S ribosomes during the initiation process is depicted in Fig. 6 by *Reactions 1* and *2*. This hypothesis is consistent with the observation that initiation factors during isolation are found bound only to native 30 S ribosomes and are not free in the supernatant (24–26).

Certain observations from other laboratories appear to be inconsistent with the mechanism just proposed. For example, it has been shown that IF-2 can form a ternary complex with GTP and fMet-tRNA (27, 28). It has been suggested that this ternary complex then binds to a mRNA-IF-3-IF-1-30 S ribosomal complex to form the complete 30 S initiation complex. Thus there are two alternate pathways of IF-2 action: binding first to 30 S ribosomes and then to fMet-tRNA; or binding first to fMet-tRNA and then to 30 S ribosomes. Work is in progress to determine which of these alternatives is correct.

At saturating concentrations of all three initiation factors, stable, unfixed IF-2 binding to 30 S ribosomes approaches a plateau value of 0.7 IF-2 molecules per 30 S ribosomes. Since only 70% of the subunits are active in fMet-tRNA binding, the simplest interpretation is that IF-2 binds stoichiometrically to

[5] J. W. B. Hershey, unpublished results.

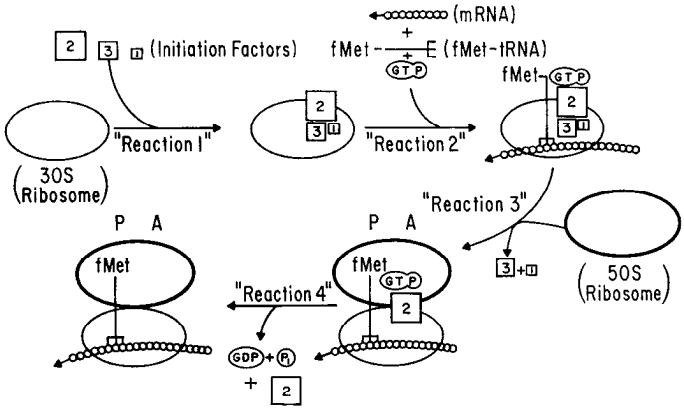

FIG. 6. Proposed model for the mechanism of initiation of protein synthesis. *Reaction 1*, binding of the three initiation factors to the 30 S ribosome. *Reaction 2*, binding of mRNA, fMet-tRNA, and GTP to form the complete 30 S initiation complex. *Reaction 3*, junction of the 50 S ribosome with the 30 S initiation complex to form an unreactive 70 S complex, with the simultaneous ejection of IF-1 and IF-3. *Reaction 4*, hydrolysis of GTP, ejection of IF-2, GDP, and P_i, and formation of a reactive fMet-tRNA-70 S complex.

active 30-S ribosomes. The correlation between IF-2 binding and fMet-tRNA binding activities suggests that the inability to bind fMet-tRNA is due to the failure to bind IF-2. In contrast, IF-3 binds well to 30 S ribosomes which are relatively inactive in the fMet-tRNA binding assay.[6]

When bound IF-2 is fixed with glutaraldehyde before sucrose gradient centrifugation, more than 1 IF-2 molecule is fixed to the ribosome. It appears that the saturation curve (with fixation) approaches a bound IF-2 to ribosome ratio of 2. The presence or absence of IF-1 and IF-3 does not affect the saturation curve. It is not known whether this apparent second binding site is simply nonspecific or whether it plays a functional role in the initiation process. Evidently, considerable care must be taken in interpreting binding date obtained by using fixation techniques.

IF-2 remains bound to the 70 S ribosome following the junction of the 50 S ribosome with the 30 S initiation complex, and leaves only after GTP hydrolysis occurs. This is the expected behavior of a factor which exhibits GTPase activity only in the presence of 70 S ribosomes (2–4). The behavior of IF-2 is unlike that of IF-1 and IF-3, both of which leave the ribosome at the subunit junction step before GTP hydrolysis occurs (5–7). Similar results for the binding and release of IF-2 have been obtained recently by Lockwood *et al.* (29).

The results cited above provide explanations for a number of phenomena observed in studies of the initiation process.

1. GTP hydrolysis is required before fMet-tRNA can react with puromycin (10) or the next aminoacyl-tRNA (30). This is consistent with the present demonstration that IF-2 remains bound to the 70 S ribosome when GTP hydrolysis is prevented, and is released only after GTP hydrolysis proceeds. The presence of IF-2 on the surface of the 70 S ribosome presumably blocks the binding or reaction of puromycin or aminoacyl-tRNA. Although the binding of the fMet-tRNA is in the P site (31, 32), the IF-2 protein is relatively large and can be expected to overlap the A site also. Thus the GTPase activity of IF-2 is functionally similar to that of elongation factor EF-Tu (33); *i.e.*, both GTP hydrolysis steps involve the ejection of the respective factors.

[6] R. Heimark, unpublished results.

Our results are in agreement with, and explain by the direct measurement of IF-2, the recent results and interpretations of Benne and Voorma (34).

2. IF-2 acts catalytically in the presence of GTP, but not $Gpp(CH_2)p$, in the binding of fMet-tRNA to 70 S ribosomes (17, 34–36). Since IF-2 is released from the 70 S ribosome after GTP hydrolysis, it is free to recycle through the initiation process, and can therefore cause the binding of more than 1 mole of fMet-tRNA per mole of IF-2 present. With $Gpp(CH_2)p$, on the other hand, only one cycle of binding can occur and the IF-2 is held bound to the 70 S complex. Our results show that with $Gpp(CH_2)p$, fMet-tRNA and IF-2 are indeed present in equal proportions on the 70 S ribosome. With 30 S ribosomes, however, IF-2 does not recycle and fMet-tRNA binding proceeds as well with $Gpp(CH_2)p$ as with GTP.

3. GTP hydrolysis *per se* is not required for the release of IF-2. Dubnoff *et al.* (17) have shown that the 30 S initiation complex depleted of GTP will react in the presence of 50 S ribosomes to form fMet-puromycin. This can be interpreted to mean that in the complete absence of GTP, IF-2 can leave the 70 S complex. That this is so is evident from the fact that when $Gpp(CH_2)p$ was included in the binding reaction (described in Fig. 5*A*), but was omitted from the gradient, IF-2 no longer was found in the 70 S region; the ^{32}P-IF-2 profile resembled instead that obtained with GTP. Since the presence of GTP causes an increase in the rate of fMet-puromycin formation (10), the role of GTP hydrolysis in the initiation process may be to increase the rate at which IF-2 leaves the 70 S complex.

The ribosomal binding and release of initiation factors during the events of initiation of protein synthesis, as derived from the studies described here and from others, are summarized in Fig. 6. All three factors are present in the complete 30 S initiation complex, but only IF-2 remains bound to the 70 S ribosome after the junction step (*Reaction 3*) and does not leave the ribosome until GTP hydrolysis occurs (*Reaction 4*). The mechanism proposed here is in substantial agreement with those suggested by Benne and Voorma (34), Lockwood *et al.* (29), and Mazumder (37). The role of GTP in the process may be not only to stimulate by hydrolysis the ejection of IF-2 from the 70 S ribosome, but also may be to catalyze the rates of binding of the initiation factors to the 30 S ribosome.

Acknowledgments—We thank R. R. Traut, A. Bollen, and S. Langberg for assistance in the preparation of the manuscript, and A. H. Lockwood for communicating before publication his results on the interaction of IF-2 and ribosomes.

REFERENCES

1. LUCAS-LENARD, J., AND LIPMANN, F. (1971) *Ann. Rev. Biochem.* **40**, 409–448
2. KOLAKOFSKY, D., DEWEY, K. F., HERSHEY, J. W. B., AND THACH, R. E. (1968) *Proc. Nat. Acad. Sci. U. S. A.* **61**, 1066–1070
3. KOLAKOFSKY, D., DEWEY, K., AND THACH, R. E. (1969) *Nature* **223**, 694–697
4. DUBNOFF, J. S., AND MAITRA, U. (1972) *J. Biol. Chem.* **247**, 2876–2883
5. HERSHEY, J. W. B., DEWEY, K. F., AND THACH, R. E. (1969) *Nature* **222**, 944–947
6. SABOL, S., AND OCHOA, S. (1971) *Nature New Biol.* **234**, 233–236
7. PON, C. L., FRIEDMAN, S. M., AND GUALERZI, C. (1972) *Molec. Gen. Genet.* **116**, 192–198
8. FAKUNDING, J. L., TRAUGH, J. A., TRAUT, R. R., AND HERSHEY, J. W. B. (1972) *J. Biol. Chem.* **247**, 6365–6367
9. SUNDARARAJAN, T. A., AND THACH, R. E. (1966) *J. Mol. Biol.* **19**, 74–90

10. Hershey, J. W. B., and Thach, R. E. (1967) *Proc. Nat. Acad. Sci. U. S. A.* **57,** 759–766
11. Moore, P. B., Traut, R. R., Noller, H., Pearson, P., and Delius, H. (1968) *J. Mol. Biol.* **31,** 441–461
12. Noll, M. (1972) Doctoral dissertation, Northwestern University
13. Hershey, J. W. B., Remold-O'Donnell, E., Kolakofsky, D., Dewey, K. F., and Thach, R. E. (1971) *Methods Enzymol.* **20,** 337–348
14. Eikenberry, E. F., Bickle, T. A., Traut, R. R., and Price, C. A. (1970) *Eur. J. Biochem.* **12,** 113–116
15. Zamir, A., Miskin, R., and Elson, D. (1971) *J. Mol. Biol.* **60,** 347–364
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
17. Dubnoff, J. S., Lockwood, A. H., and Maitra, U. (1972) *J. Biol. Chem.* **247,** 2884–2894
18. Nomura, M., and Lowry, C. V. (1967) *Proc. Nat. Acad. Sci. U. S. A.* **58,** 946–953
19. Bretscher, M. S. (1968) *Nature* **220,** 1233–1234
20. Revel, M., Herzberg, M., and Greenshpan, H. (1969) *Cold Spring Harbor Symp. Quant. Biol.* **34,** 261–275
21. Noll, M., and Noll, H. (1972) *Nature New Biol.* **238,** 225–228
22. Thach, S. S., and Thach, R. E. (1971) *Nature New Biol.* **229,** 219–221
23. Groner, Y., and Revel, M. (1971) *Eur. J. Biochem.* **22,** 144–152
24. Parenti-Rosina, R., Eisenstadt, A., and Eisenstadt, J. M. (1969) *Nature* **221,** 363–365
25. Miller, M. J., Zasloff, M., and Ochoa, S. (1969) *FEBS Lett.* **3,** 50–52
26. Dubnoff, J. S., Lockwood, A. H., and Maitra, U. (1972) *Arch. Biochem. Biophys.* **149,** 528–540
27. Rudland, P. S., Whybrow, W. A., and Clark, B. F. C. (1971) *Nature New Biol.* **231,** 76–78
28. Lockwood, A. H., Chakraborty, P. R., and Maitra, U. (1971) *Proc. Nat. Acad. Sci. U. S. A.* **68,** 3122–3126
29. Lockwood, A. H., Sarkar, P., and Maitra, U. (1972) *Proc. Nat. Acad. Sci. U. S. A.* **69,** 3602–3605
30. Ohta, T., Sarker, S. and Thach, R. E. (1967) *Proc. Nat. Acad. Sci. U. S. A.* **58,** 1638–1644
31. Thach, S. S., and Thach, R. E. (1971) *Proc. Nat. Acad. Sci. U. S. A.* **68,** 1791–1795
32. Kuechler, E. (1971) *Nature New Biol.* **234,** 216–218
33. Ono, Y., Skoultchi, A., Waterson, J., and Lengyel, P. (1969) *Nature* **222,** 645–648
34. Benne, R., and Voorma, H. O. (1972) *FEBS Lett.* **20,** 347–351
35. Chae, Y.-B., Mazumder, R., and Ochoa, S. (1969) *Proc. Nat. Acad. Sci. U. S. A.* **63,** 828–833
36. Remold-O'Donnell, E., and Thach, R. E. (1970) *J. Biol. Chem.* **245,** 5737–5742
37. Mazumder, R. (1972) *Proc. Nat. Acad. Sci. U. S. A.* **69,** 2770–2773