THE MECHANISM OF ACTION OF INITIATON FACTOR 3 IN PROTEIN SYNTHESIS, I. STUDIES ON RIBOSOMES CROSSLINKED WITH DIMETHYLSUBERIMIDATE

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Summary: The mechanism of action of chain initiation factor 3 in translation was examined by using E. coli 70S ribosomes which were covalently crosslinked with dimethylsuberimidate. Crosslinked ribosomes were inactive in AUG-dependent fMet-tRNA binding, and were not stimulated by IF-3 in poly(U) translation. IF-3 is known to be required for maximal rates of amino acid incorporation with synthetic polynucleotides at 18 mM Mg^{2+}. A direct interaction of IF-3 with 70S ribosomes was demonstrated by crosslinking ^{14}C-labeled IF-3 to 70S ribosomes. The labeled factor was also crosslinked to 30S and 50S ribosomal subunits. A model is presented proposing the mechanism of action of IF-3 on 70S ribosomes.

The initiation of protein synthesis in cell-free extracts of E. coli requires the participation of at least three proteins, IF-1, IF-2 and IF-3. Previous work has shown that IF-3 is essential, along with IF-1 and IF-2, for the translation of phage RNA (1,2) as well as for the ribosomal binding at 3-5 mM Mg^{2+} of fMet-tRNA in the presence of natural mRNA or ApUpG (3,4), and of acPhe-tRNA in the presence of poly(U) (5,6,7). More recently, IF-3 was found to be required at 18 mM Mg^{2+} for maximal rates of amino acid incorporation with poly(U), poly(A) and other synthetic polynucleotides (5).

In addition to its role in binding mRNA to the ribosome, IF-3 was found to promote the dissociation of 70S ribosomes into subunits at 5 mM Mg^{2+} (3,8,9). By binding directly to 30S subunits (10-14), IF-3 prevented their reassociation with 50S particles, thus providing a pool of free 30S subunits (15) which may be required for chain initiation (16). Subsequent to the formation of an initiation complex on the 30S subunit, the 50S subunit is added, and IF-3 is released (10). Some aspects of this scheme of events are not yet clearly understood. In particular, the precise state of the equilibrium between free subunits and 70S particles appears to be critically dependent on the Mg^{2+} concen-

tration of the medium in which the ribosomes are suspended. The ability of IF-3 to displace this equilibrium appears to be less effective at Mg^{2+} concentrations above 5 mM (9).

In this paper, we have attempted to elucidate the mechanism of action of IF-3 in poly(U) translation at 18 mM Mg^{2+} by studying the effect of IF-3 on crosslinked ribosomes and by demonstrating a direct interaction of the factor with 70S ribosomes.

MATERIALS AND METHODS:

Preparation of Ribosomes, Ribosomal Subunits and Chain Initiation Factors: Ribosomes were prepared from E. coli Q13 or MRE600 cells and were washed twice with 1.0 M NH_4Cl (17). The 70S particles were purified by two successive separations on 10-30% sucrose gradients in a buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl and 10 mM magnesium acetate, and contained no detectable 30S subunits. 30S and 50S ribosomal subunits were prepared from the purified 70S ribosomes according to Traub et al. (18). Ribosomes and ribosomal subunits were stored at 0° in a buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 10 mM magnesium acetate and 6 mM 2-mercaptoethanol.

IF-1, IF-2 and IF-3 were purified from the 1.0 M NH_4Cl ribosomal wash as in previous work (4,5,17). The preparation of ^{14}C-labeled IF-3 was carried out by reductive alkylation with [^{14}C]formaldehyde (59.2 mCi/mmole, NEN) as described by Gualerzi et al. (19). The purity of the labeled factor was estimated to be greater than 90% as judged by SDS-polyacrylamide gel electrophoresis, and its specific radioactivity was 9100 cpm per µg. Its activity in poly(U) translation remained unchanged after modification.

Crosslinking of Ribosomes and Crosslinking of ^{14}C-Labeled IF-3 to 30S, 50S and 70S Ribosomes. Purified 70S ribosomes were crosslinked with Me_2Sub[1] as previously described (20). For the crosslinking of IF-3 to ribosomes, two equivalents of [^{14}C]H_3-IF-3 were incubated for 15 min at 0° with ribosomes or ribosomal subunits (1mg/ml final concentration) in Buffer I (20 mM N,N-bis(2-hydroxyethyl)glycine (Bicine), pH 8.5, 10 mM magnesium acetate, 100 mM KCl). A solution of 0.1 M Me_2Sub in Buffer I containing

[1]Abbreviations: Me_2Sub, dimethylsuberimidate; CLR, crosslinked ribosomes, i.e., 30S-50S complexes covalently joined by treatment with Me_2Sub.

0.2 M KOH was added to the above mixture to give a final Me_2Sub concentration of 10 mM. The reaction mixture was incubated for 2 hr at 0° after which 0.1 volume of 1.0 M Tris-HCl, pH 7.0, was added in order to stop the reaction. After dialysis at 4° for several hours against a buffer containing 10 mM Tris-HCl, pH 7.5 10 mM magnesium acetate and 1.0 M NH_4Cl (Buffer II), the ribosomes were centrifuged for 3 hr at 65,000 rpm in the Spinco 65 rotor. The ribosomal pellets were suspended in Buffer II, recentrifuged to remove any non-covalently bound IF-3, and finally suspended in Buffer III (10 mM Tris-HCl, pH 7.5, 0.5 mM magnesium acetate, 100 mM NH_4Cl). Crosslinked [^{14}C]H_3-IF-3-65S particles were further purified by centrifugation at 27,000 rpm on 5-25% sucrose density gradients in Buffer III in a Spinco SW27 rotor. The crosslinked 30S subunits (Fig. 2A) migrated as a single peak at 30S before crosslinking.

RESULTS:

Ribosomes crosslinked with Me_2Sub were used to study the nature of the interaction of IF-3 with 70S particles. CLR do not dissociate at 0.5 mM Mg^{2+} and are composed of covalently joined 30S and 50S subunits. These particles are able to translate poly(U) at approximately 80% of the activity of unmodified ribosomes, yet are unable to translate natural mRNA, or participate in initiation complex formation with f2 RNA (20). As illustrated in Table I, AUG-dependent binding of fMet-tRNA with the ribosomes from either *E. coli* MRE600 or Q13 was inhibited 75 to 84% when the ribosomes were crosslinked with Me_2Sub. The relative stimulation by the purified initiation factors was also markedly decreased. It was previously shown that ribosomal subunits crosslinked separately with Me_2Sub were stimulated by initiation factors in the translation of natural mRNA (20).

The exposure of 70S ribosomes to Me_2Sub might conceivably have affected several structural proteins, and consequently decreased the affinity of IF-3 for a ribosomal site. In order to rule out this possibility, both control ribosomes and CLR were assayed in poly(U) translation with increasing levels of IF-3 (Fig. 1). Control ribosomes were stimulated 15-fold by the addition of IF-3, but CLR were only slightly stimulated even at concentrations of factor far greater than the normal saturation level for translation of poly(U).

TABLE I

AUG-dependent binding of f[^{14}C]Met-tRNA to ribosomes: Effect of crosslinking on stimulation by initiation factors

Ribosomes	Initiation factors IF-1+IF-2+IF-3	f[^{14}C]Met-tRNA bound	Stimulation by initiation factors
		pmoles	x fold
Experiment A			
70S	-	0.33	-
70S	+	3.60	10.9
70S CLR	-	0.33	-
70S CLR	+	0.57	1.7
Experiment B			
70S	-	0.33	-
70S	+	3.60	10.9
70S CLR	-	0.33	-
70S CLR	+	0.64	1.9

Each incubation contained, in 0.05 ml, 0.10 M NH_4Cl; 0.05 M Tris-HCl, pH 7.2; 5 mM magnesium acetate; 1.0 mM DTT; 0.2 mM GTP; 1 nmole of AUG; 18 pmoles of f[^{14}C]Met-tRNA (221 mCi per mmole); and in Expt. A, 1.15 A_{260} units of *E. coli* MRE600 ribosomes or 1.0 A_{260} unit of CLR from *E. coli* MRE600; in Expt. B, 1.26 A_{260} units of *E. coli* Q13 ribosomes or 1.15 A_{260} units of CLR from *E. coli* Q13; 2.6 µg of IF-1; 0.15 µg of IF-2; 2.0 µg of IF-3. Incubation was for 15 min at 25°, and the reaction was terminated as previously described (4).

It was therefore of interest to demonstrate a direct interaction of IF-3 with 70S ribosomes. We have approached this problem by incubating either 70S, 50S or 30S particles with [^{14}C]H_3-IF-3 and subsequently crosslinking the complexes with Me_2Sub. The results of one such experiment are illustrated in Fig. 1. IF-3 was found to interact with each form of particle and could be

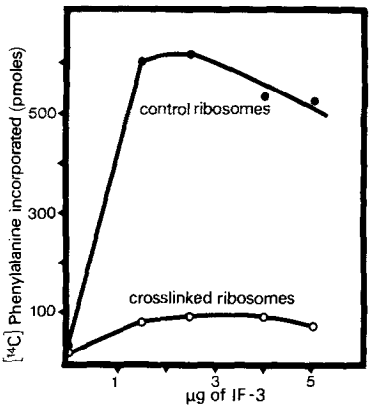
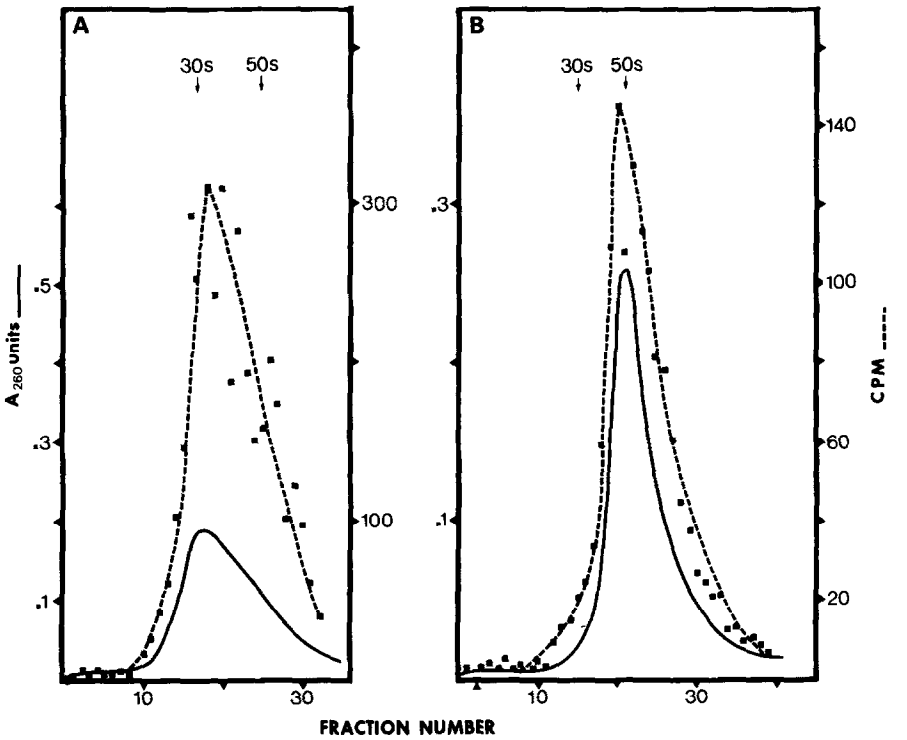


Figure 1. Poly(U) translation as a function of IF-3 concentration. Phenylalanine incorporation was determined as in previous work (5). Each assay contained 2.0 µg of poly(U). Blank values in the absence of poly(U) were subtracted from the values shown. —●—●—, 70S ribosomes from E. coli MRE600, 1.5 A_{260} units; —○—○—, CLR from E. coli MRE600, 2.2 A_{260} units.

crosslinked in place. The crosslinked dimers, which migrate as 65S (20) contain approximately twice as much IF-3 per ribosome as do the purified subunits (0.26 copy of IF-3 per 30S or 50S subunit, and 0.43 copy of IF-3 per 65S particle).

DISCUSSION:

Previous studies have indicated that IF-3 is capable of dissociating 70S ribosomes into subunits when the Mg^{2+} concentration is below 6.7 mM (9). However, Mg^{2+} concentrations that are required for cell-free protein synthesis with either natural or synthetic mRNAs (5) are greater than those which permit IF-3-mediated dissociations of ribosomes. Recently, Zitomer and Flaks (21) have measured the equilibrium constant for the association of ribosomal subunits into intact ribosomes. The high value for the calculated K_{assoc} at 10 mM Mg^{2+} would seem to preclude dissociation of 70S ribosomes by IF-3 at 18 mM Mg^{2+}. Evidence is also emerging which indicates that IF-3 can interact with 50S and 70S particles. Vermeer et al. (22) demonstrated an association of IF-3 with 70S ribosomes on sucrose density gradients after incubation of ribosomes with the factor and fixation with glutaraldehyde. The mobility of 50S subunits and 70S ribosomes in the presence of IF-3 was shown to be reduced in polyacrylamide gel electrophoresis, possibly indicating an IF-3 induced conformational change of the particles (22,23).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A260 units
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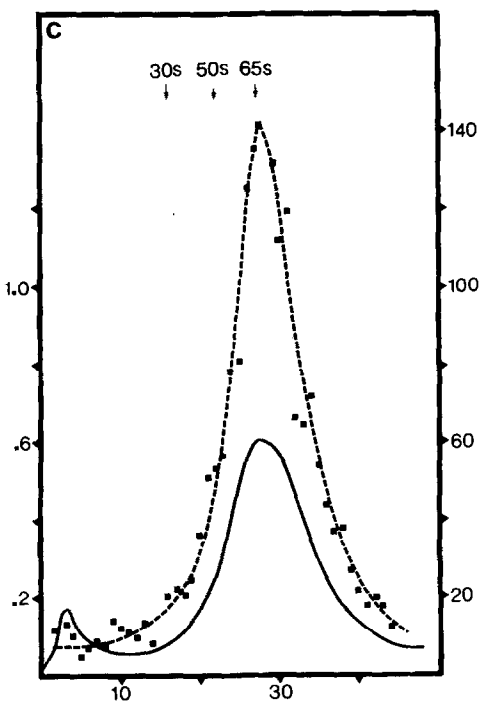
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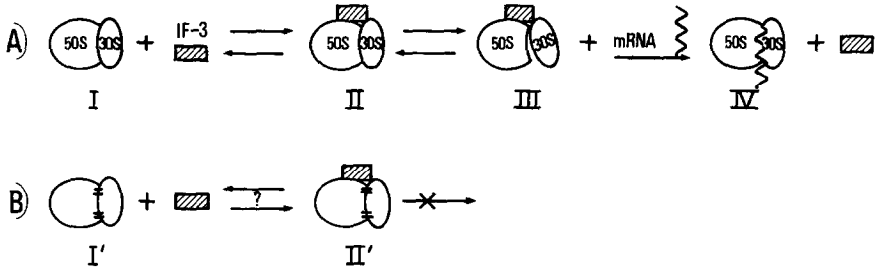

Figure 3. Model for the mechanism of action of IF-3 on 70S ribosomes. IF-3 binds to 70S ribosomes and is in contact with both subunits (A-II). This causes a structural loosening (A-III) which exposes the proper message binding site. mRNA then binds to the 70S and IF-3 is released (A-IV). When the subunits are covalently joined by crosslinking (B-I) IF-3 cannot cause the structural loosening required for exposure of the mRNA binding site.

In this paper we would like to suggest that stimulation of poly(U) translation by IF-3 at 18 mM Mg^{2+} is due to a direct interaction of the factor with 70S ribosomes. Such an interaction was demonstrated by crosslinking the factor in place on 70S ribosomes as well as on 30S and 50S subunits. The increased binding to the 65S dimer over that to the subunits is expected since it is evident that IF-3 can bind to both the free 30S and 50S particles. The IF-3 binding site on the 70S ribosome may be either a shared binding site by the two particles or two separate binding sites, one on each subunit. In either case, IF-3 has physical contact with both halves of the 70S ribosome and hence, an increased probability of making as least one crosslink between the factor and the 70S ribosome over that of making a crosslink with a subunit alone. The fact that IF-3 binding to 50S subunits has not been generally observed at 5 mM Mg^{2+} or above may be due to the sucrose density gradient technique used by others (12).

Normal and crosslinked ribosomes were used in IF-3-mediated

Figure 2. Sucrose density gradients of ribosomes crosslinked in the presence of IF-3. The 30S, 50S and 65S particles bound 0.26, 0.26 and 0.43 copy of IF-3 per particle, respectively. Aliquots of the crosslinked ribosomes, as noted below, were applied to the top of 5 ml 5-25% sucrose density gradients in 10 mM Tris-HCl, pH 7.5, 0.5 mM Mg^{2+} and 100 mM NH_4Cl. They were centrifuged at 50,000 rpm for 75 min in a Spinco SW 50.1 rotor and fractionated using a Buchler AutoDensiflow and a Gilford flow cell. The fractions were counted in Aquasol for 10 min in a Packard Tri-Carb scintillation counter. (A) 1.87 A_{260} units of 30S subunits, (B) 0.87 A_{260} unit of 50S subunits, (C) 1.83 A_{260} units of 65S ribosomes.

poly(U) translation. IF-3 stimulated normal ribosomes but had little effect on CLR, although both preparations of ribosomes had approximately equal activities in the absence of the factor. It seems likely that IF-3 must cause a conformational change in the 70S structure to allow proper message binding. As has been previously demonstrated, IF-1 may participate in this process (5). The nature of this structural change may involve an opening at the 30S-50S interface, as if the subunits were hinged together. After crosslinking the topography of CLR is fixed and IF-3 cannot open the 70S structure to allow proper message binding. This proposed model is depicted in Fig. 3.

The identification of specific crosslinks between IF-3 and ribosomal proteins from either the 30S or 50S subunit is currently under investigation (24).

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