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EFFECT OF POLYPEPTIDE INITIATION FACTORS ON THE SPERMIDINE STIMULATION OF INITIATION COMPLEX FORMATION

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SUMMARY: The AUG- and MS2 RNA-dependent fMet-tRNA binding to 30S ribosomal subunits was stimulated by spermidine with any individual or combination of initiation factors capable of participating in the formation of an initiation complex. When 70S ribosomes were used instead of 30S ribosomal subunits, IF-3 was necessary for spermidine stimulation of the complex formation.

It is well known that polyamines have not only a sparing effect on the Mg^{2+} requirement for polypeptide synthesis but also a stimulating effect, which can not be fulfilled by any amount of Mg^{2+} (1-6). In addition, it has been reported that the stimulation of polypeptide synthesis by polyamines is due mainly to a stimulation of the initiation complex formation of polypeptide synthesis (3-6).

In this communication, we will show that the ability of IF-3 to dissociate 70S ribosomes into 30S and 50S ribosomal subunits is important for the spermidine stimulation of initiation complex formation.

MATERIALS AND METHODS

Materials - The incubated 30,000 x g supernatant (IS-30) of E. coli Q13 and unwashed ribosomes were prepared according to the method of Nirenberg and Matthaei (7). Washed ribosomes and 100,000 x g supernatant treated with Sephadex G-50 (S-S100) were prepared as described previously (1) except that 1 M NH_4Cl was used instead of 0.5 M NH_4Cl for washing the ribosomes. The preparation of ribosomal subunits (30S and 50S) and of crude initiation factors was carried out according to the procedure of Igarashi et al. (8). Transfer RNA was prepared from the 100,000 x g supernatant by the method of Zubay (9), except for the omission of the 2-propanol treatment. MS2 RNA was prepared from the MS2 phage according to the method described by Gierer

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and Schramm (10). The preparation of AUG was carried out according to the procedure of Thach et al. (11). The method of Nakamoto and Kolakofsky (12) was adopted for the preparation of f[^{3}H]Met-tRNA by using 2 μM [^{3}H]methionine (specific activity, 3.3 Ci/mmol, New England Nuclear).

Preparation of initiation factors - Each initiation factor was purified from a crude initiation factor preparation of *E. coli* Q13 with some modifications of the method of Benne et al. (13). IF-2 activity was precipitated between 0 and 50% saturation with ammonium sulfate. The precipitate was dissolved in Buffer I (20 mM Tris-HCl, pH 7.4, 7 mM 2-mercaptoethanol, and 10% glycerol) containing 150 mM KCl and absorbed on a column of DEAE-Sephadex A-50. The IF-2 fraction was eluted with Buffer I containing 300 mM KCl and dialyzed against Buffer I containing 50 mM KCl. This preparation was applied to a column of phosphocellulose and IF-2 was eluted with Buffer I containing 600 mM KCl. IF-1 and IF-3 activities were precipitated between 50 and 70% saturation with ammonium sulfate. The precipitate was dissolved in Buffer II (30 mM Tris-HCl, pH 7.4, 1 mM EDTA, 6 mM 2-mercaptoethanol, and 10% glycerol) and applied to a column of DEAE-Sephadex A-50. The IF-1 fraction was eluted with Buffer II, while IF-3 activity was eluted with Buffer II containing 150 mM KCl and dialyzed against Buffer III (10 mM Tris-HCl, pH 7.4, 1 mM magnesium acetate, 250 mM NH_4Cl, 1 mM dithiothreitol, and 10% glycerol). This fraction was applied to a column of phosphocellulose and IF-3 was eluted with Buffer III containing 700 mM NH_4Cl instead of 250 mM NH_4Cl. The assays of initiation factors were performed by the method of Wahba and Miller (14). Protein content was determined according to the method of Lowry et al. (15).

Assay of fMet-tRNA binding to 70S ribosomes or 30S ribosomal subunits - The reaction mixture (0.1 ml), which contained 50 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 1 mM dithiothreitol, 1 mM GTP, 24,000 cpm of f[^{3}H]Met-tRNA (20 μg of tRNA), 1 A_{260} unit of 1 M NH_4Cl-washed ribosomes or 0.5 M NH_4Cl-washed 30S ribosomal subunits, 6 μg of AUG or 15 μg of MS2 RNA, 2 μg each of IF-1, IF-2, and IF-3, and magnesium acetate and spermidine at the specified concentrations, was incubated at 25° C for 10 min. The amount of f[^{3}H]Met-tRNA bound to 70S ribosomes or 30S ribosomal subunits was measured by the procedure of Nirenberg and Leder (16).

Sucrose gradient centrifugation analysis of the initiation complex of MS2 RNA, f[^{3}H]Met-tRNA and ribosomes - After incubation of 0.4 ml of the above mentioned reaction mixture at 25° C for 10 min, 20% glutaraldehyde neutralized with sodium hydroxide was added to make a final concentration of 0.25% (13). To analyze the initiation complex, a 0.37 ml aliquot of the reaction mixture was placed on the top of a 5 to 20% sucrose gradient (4.7 ml) in 10 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 1 mM dithiothreitol, and magnesium acetate and spermidine at the specified concentrations. The tube was centrifuged in a Hitachi RPS-50 rotor for 135 min at 37,000 rpm. After centrifugation, twelve-drop fractions were collected from the bottom of the tube. Absorbance at 260 nm in each fraction was measured after a 13-fold dilution. The bound f[^{3}H]Met-tRNA was measured by the procedure of Nirenberg and Leder (16) using a 0.3 ml aliquot of each fraction.

RESULTS

Effect of spermidine and initiation factors on initiation complex formation - The AUG- and MS2 RNA-dependent fMet-tRNA

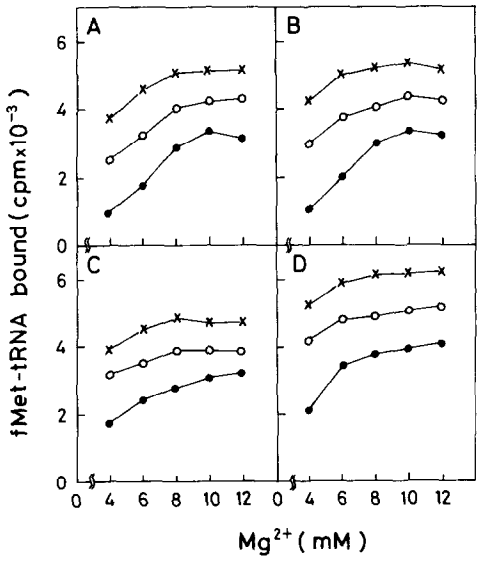

Fig. 1. Effect of spermidine and initiation factors on AUG-dependent fMet-tRNA binding to 30S ribosomal subunits. The assay was carried out as described in Materials and Methods.
A. IF-2; B. IF-1 and IF-2; C. IF-2 and IF-3; D. IF-1, IF-2 and IF-3. ●——●, no spermidine; o——o, 0.75 mM spermidine; x——x, 1.5 mM spermidine.

binding to 30S ribosomal subunits was stimulated by spermidine with IF-2 and several combinations of initiation factors, as shown in Fig. 1 and Table 1, respectively. The optimal concentration of spermidine for the stimulation of MS2 RNA-dependent fMet-tRNA binding was 1.5 to 2.5 mM (Table 1). When AUG was used as a template, a similar optimal concentration range of spermidine was obtained. In addition, the degree of stimulation by 1.5 mM spermidine (1.3- to 1.4-fold) of initiation complex formation was nearly equal to that observed with 2 mM spermidine for MS2 RNA-dependent polypeptide synthesis with the combination of 30S and 50S subunits (6).

As shown in Fig. 2 and Table 2, respectively, AUG-and MS2 RNA-dependent fMet-tRNA binding to 70S ribosomes was stimulated by spermidine only when IF-3 was added to the reaction mixture

Table 1. Effect of spermidine and initiation factors on MS2 RNA-dependent fMet-tRNA binding to 30S ribosomal subunits.

Factors added	Ions (mM)		fMet-tRNA bound (cpm)	Stimulation by spermidine (-fold)
	Mg^{2+}	Spermidine		
IF-2	10	-	356	-
"	10	0.75	442	1.24
"	10	1.5	494	1.39
"	8	2.5	500	1.40
IF-1 + IF-2	10	-	874	-
"	10	0.75	1036	1.19
"	10	1.5	1218	1.39
"	8	2.5	1198	1.37
IF-2 + IF-3	10	-	1090	-
"	10	0.75	1324	1.21
"	10	1.5	1536	1.41
"	8	2.5	1480	1.36
IF-1 + IF-2 + IF-3	10	-	1329	-
"	10	0.75	1606	1.21
"	10	1.5	1866	1.40
"	8	2.5	1870	1.41

The assay was carried out as described in Materials and Methods. Magnesium ions specified in the table were at the concentration found to be optimal for initiation complex formation according to prior testing of four Mg^{2+} concentrations at given spermidine concentrations.

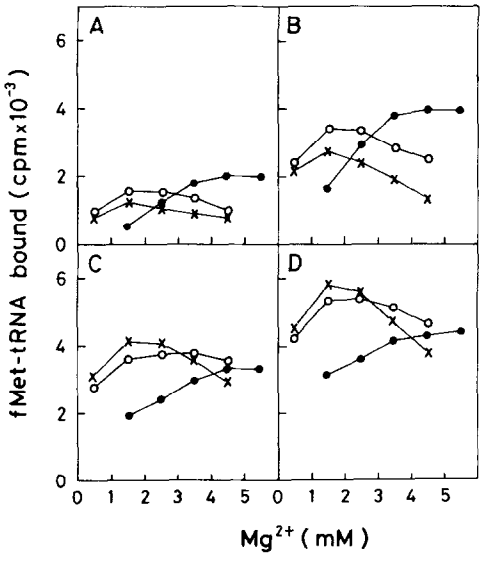

Fig. 2. Effect of spermidine and initiation factors on AUG-dependent fMet-tRNA binding to 70S ribosomes. The assay was carried out as described in Materials and Methods. A. IF-2; B. IF-1 and IF-2; C. IF-2 and IF-3; D. IF-1, IF-2 and IF-3. ●——●, no spermidine; o——o, 0.375 mM spermidine; x——x, 0.75 mM spermidine.

Table 2. Effect of spermidine and initiation factors on MS2 RNA-dependent fMet-tRNA binding to NH_4Cl washed 70S ribosomes.

Factors added	Ions (mM)		fMet-tRNA bound	Stimulation by spermidine
	Mg^{2+}	Spermidine	(cpm)	(-fold)
IF-2	2.5	-	521	-
"	1.5	0.375	360	0.69
"	1.5	0.75	318	0.61
"	1.0	1.5	261	0.50
IF-1 + IF-2	1.5	-	863	-
"	0.5	0.375	639	0.74
"	0.5	0.75	561	0.65
"	0.5	1.5	480	0.56
IF-2 + IF-3	2.5	-	894	-
"	1.5	0.375	948	1.06
"	1.5	0.75	1086	1.21
"	1.0	1.5	1025	1.15
IF-1 + IF-2 + IF-3	3.5	-	1935	-
"	2.5	0.375	2123	1.10
"	1.5	0.75	2547	1.32
"	1.0	1.5	2365	1.22

The assay was carried out as described in Materials and Methods. The magnesium ion concentrations specified were optimal on the basis described in Table 1.

in addition to some other initiation factor(s). When IF-2 or the combination of IF-1 and IF-2 was present, spermidine inhibited the formation of an initiation complex. However, when the combinations of IF-2 and IF-3 or IF-1, IF-2 and IF-3 were added to the reaction mixture, spermidine not only caused a shift of the Mg^{2+} concentration to a lower optimum, but also they stimulated the formation of the initiation complex. The optimal concentration of spermidine for stimulation was 0.75 mM. A further increase of spermidine concentration gradually inhibited the initiation complex formation (Table 2). The presence of IF-3 increased the spermidine concentration required for maximal formation of initiation complex (Fig. 2 and Table 2). In order

Table 3. Effect of initiation factors on the stimulation of AUG-dependent fMet-tRNA binding to NH_4Cl washed 70S ribosomes.

Changing initiation factor	Ions (mM)		fMet-tRNA bound (cpm)	Stimulation by spermidine (-fold)
	Mg^{2+}	Spermidine		
IF-3 (2 μg)	3	-	5595	
	1.5	0.75	6247	1.12
IF-3 (4 μg)	3	-	4670	
	1.5	0.75	5598	1.20
IF-3 (6 μg)	3	-	3845	
	1.5	0.75	5676	1.48
IF-2 (0.75 μg)	3	-	3002	
	1.5	0.75	3364	1.12
IF-2 (1.5 μg)	3	-	4769	
	1.5	0.75	5295	1.11
IF-2 (3 μg)	3	-	6057	
	1.5	0.75	6674	1.10
IF-1 (1 μg)	3	-	5078	
	1.5	0.75	5613	1.11
IF-1 (3 μg)	3	-	5414	
	1.5	0.75	6295	1.16

The assay was carried out as described in Materials and Methods except that the amount of one initiation factor was changed as indicated in the table. The magnesium ion concentrations specified were optimal on the basis described in Table 1.

to confirm that IF-3 is necessary for the spermidine stimulation of the formation of 70S ribosome initiation complex, the amount of one initiation factor was changed in the presence of a constant amount of the other two initiation factors (Table 3). When the amount of IF-1 or IF-2 was changed, the degree of spermidine stimulation of initiation complex formation did not change significantly. However, the degree of spermidine stimulation increased gradually as the amount of IF-3 was increased.

Sedimentation behavior of the complex of ribosomes, MS2 RNA and fMet-tRNA - In the absence of spermidine, both 70S and 30S

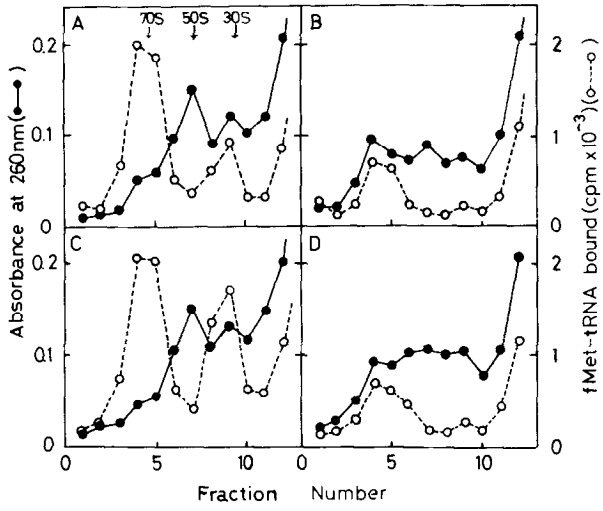

Fig. 3. Sedimentation behavior of the complex of ribosomes, MS2 RNA and fMet-tRNA. Sucrose gradient centrifugation and measurement of bound fMet-tRNA to ribosomes were carried out as described in Materials and Methods.
A. IF-1, IF-2 and IF-3 were added and Mg^{2+} was 5 mM;
B. IF-1 and IF-2 were added and Mg^{2+} was 5 mM;
C. IF-1, IF-2 and IF-3 were added and Mg^{2+} and spermidine were 1.5 mM and 0.75 mM, respectively;
D. IF-1 and IF-2 were added and Mg^{2+} and spermidine were 1.5 mM and 0.75 mM, respectively.

initiation complex formation was stimulated by IF-3 at 5 mM Mg^{2+} (Fig. 3A and 3B). In the presence of 0.75 mM spermidine and 1.5 mM Mg^{2+}, IF-3 also stimulated both 70S and 30S initiation complex formation (Fig. 3C and 3D). When the formation of the initiation complexes was compared with and without spermidine (Fig. 3A and 3C), stimulation of fMet-tRNA binding by spermidine occurred primarily on the 30S ribosomal subunit region rather than on the 70S ribosome region.

DISCUSSION

The data presented in the Results section show that 50S ribosomal subunits disturb the spermidine stimulation of the formation of an initiation complex unless IF-3 is present.

The IF-3 is not only required for the binding of natural mRNA (17,18), but it also dissociates 70S ribosomes into 30S

and 50S subunits (19,20). Our data suggest that this dissociation activity by IF-3 may play an important role in the spermidine stimulation of polypeptide synthesis.

Since 50S ribosomal subunits inhibit the functional interaction between 30S subunits and phage RNA (21), the disturbance by 50S subunits of spermidine stimulation of the formation of initiation complex may occur through the inhibition of the binding of mRNA to 30S subunits.

IF-1 has been reported to stimulate the IF-3 mediated dissociation of 70S ribosomes into 30S and 50S subunits (22,23). This function of IF-1 also can be inferred from our experimental data. Although IF-1 did not increase the degree of spermidine stimulation of the formation of the 30S initiation complex in the presence of IF-2 and IF-3 (Fig. 1 and Table 1), IF-1 increased the degree of spermidine stimulation of the formation of initiation complex with 70S ribosomes in the presence of IF-2 and IF-3 (Fig. 2 and Table 2).

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