THE EFFECTS OF INITIATION FACTOR 3 ON THE FORMATION OF 30S INITIATION COMPLEXES WITH SYNTHETIC AND NATURAL MESSENGERS

D. Parker Suttle and Joanne M. Ravel

Clayton Foundation Biochemical Institute and Department of Chemistry
The University of Texas at Austin, Austin, Texas 78712

Received February 1 1974

Initiation factor IF-3 is required for the binding of fMet-tRNA to 70S ribosomes directed by AUG, poly (U,G), f_2RNA and T_4 late RNA as well as for the binding of acPhe-tRNA directed by poly (U). In contrast, IF-3 is not required for the binding of the initiator aminoacyl-tRNAs to isolated 30S subunits directed by the synthetic messengers, but is required for maximal formation of initiation complexes with natural messengers. These data indicate that with synthetic messengers the sole function of IF-3 is to dissociate the 70S ribosomes into subunits, whereas with natural messengers IF-3 is required not only for dissociation of the ribosomes but also for the binding of the messenger to the 30S subunit.

In a previous investigation (1) we reported that initiation factor 3 (IF-3) is required for the poly (U)-directed binding of acPhe-tRNA to 70S ribosomes as well as for the binding of fMet-tRNA to 70S ribosomes directed by AUG, poly (U,G) and f_2RNA. Similar results have been reported by other investigators using unfractionated ribosomes from various strains of Escherichia coli (2-6). In an early report Wahba et al. (2) showed that IF-3 did not stimulate the AUG-dependent binding of fMet-tRNA to isolated 30S ribosomal subunits. The results of this investigation show that IF-3 is not required for binding of fMet-tRNA to isolated 30S subunits directed by either AUG or poly (U,G), nor is it required for poly (U)-directed binding of acPhe-tRNA to 30S subunits. However, with natural messengers such as f_2RNA and T_4 late RNA, IF-3 is required for maximal binding of fMet-tRNA to isolated 30S subunits. Similar results have been obtained recently by Ochoa and his collaborators (personal communication). These data indicate that with 70S ribosomes and synthetic messengers the IF-3 requirement observed in vitro can be attributed solely to its dissociation factor (7, 8)

activity and not to its ability to recognize specific nucleotide sequences. With natural messengers IF-3 is required not only to dissociate the ribosomes but also to promote the formation of 30S initiation complexes.

EXPERIMENTAL

Materials: ac[^{14}C]Phe-tRNA and f[^{3}H]Met-tRNA were prepared as previously described (9, 1). Poly (U), poly (U,G) and AUG were purchased from Miles Laboratories. Bacteriophage f_2RNA and T_4 late RNA (isolated 17 minutes after infection) were kindly supplied by Mr. Michael Haralson. Salt-washed ribosomes were prepared from E. coli W cells as previously described (10). The ribosomes were dissociated into subunits by dialysis against buffer containing 20 mM Tris-HCl, pH 7.5; 1 mM $MgCl_2$; 0.1 M NH_4Cl; and 1 mM dithiothreitol (DTT). The subunits were isolated by density gradient centrifugation for 4.5 hours at 48,000 rpm in the Beckman Ti14 zonal rotor using a 10-30% linear sucrose gradient in the same buffer. The isolated subunits were pelleted by centrifugation in the Beckman 60Ti rotor for 12 hours at 60,000 rpm. The subunits were resuspended in a small amount of buffer containing 20 mM Tris, pH 7.5; 10 mM $MgCl_2$; 50 mM NH_4Cl; 1 mM DTT; 0.5 mM EDTA; and 20% glycerol and were stored at -70°C.

Assays: fMet-tRNA Binding. The standard reaction mixture contained in a total volume of 0.15 ml: Tris-HCl, pH 7.7, 50 mM; DTT, 5 mM; NH_4Cl, 0.1 M; $MgCl_2$, 5 mM; bovine serum albumin, 0.15 mg; tRNA, 0.4 A_{260} units, charged with 20 pmoles of [^{3}H]-methionine of which approximately 50% were formylated; GTP, 0.2 mM; IF-1, IF-2, IF-3, as indicated; either 0.6 A_{260} units of ribosomes containing approximately 60-70% 70S couples or 0.18 A_{260} units of 30S subunits; and either 0.1 A_{260} units of AUG, 0.2 A_{260} units of poly (U,G), 2.7 A_{260} units of f_2RNA, or 0.67 A_{260} units of T_4 late RNA.

acPhe-tRNA Binding. The standard reaction mixture was modified to contain: 1.0 A_{260} unit of tRNA charged with 30 pmoles of ac[^{14}C]-phenylalanine; 0.1 mM GTP; and 10 μg of poly (U). The times and temperatures of the incubations

are given in the legends to the tables, and the amounts of f[^{3}H]Met-tRNA and ac[^{14}C]Phe-tRNA bound to the ribosomes were determined by the Millipore filter method as previously described (9).

Separation and Purification of Initiation Factors: Initiation factors IF-1, IF-2, and IF-3 were isolated from the 0-80% ammonium sulfate fraction of the 1 M NH_4Cl wash of E. coli Q13 ribosomes by chromatography on DEAE cellulose followed by chromatography on phosphocellulose as previously described (1). Phosphocellulose preparations (PC I) of IF-3 and IF-2 were further purified in the following manner. Approximately 25 ml of pooled PC I preparations of IF-3 derived from 300 g E. coli Q13 cells were dialyzed for 4 hours at 4^{o} against Buffer D (50 mM Tris-HCl, pH 7.5; 0.5 mM DTT; 1 mM EDTA; and 10% glycerol) containing 0.2 M NH_4Cl, and applied to a 4 cc phosphocellulose column previously equilibrated in the same buffer. The column was washed with 20 ml of Buffer D containing 0.2 M NH_4Cl or until the A_{280} reading was nil. The IF-3 was then eluted by washing the column with Buffer D containing 0.6 M NH_4Cl. The majority of the activity was recovered in 2-3 ml. The PC II IF-3 preparation contained approximately 0.7 mg per ml protein and was stored in small aliquots at -70^{o}C. SDS gel electrophoresis showed only 1 major band and 2 very minor ones. Approximately 80 ml of pooled PC I preparations of IF-2 derived from 300 g of E. coli Q13 cells were dialyzed against Buffer D containing 0.1 M NH_4Cl as described above, and applied to a 4 cc phosphocellulose column previously equilibrated in the same buffer. The column was washed with 20 ml of Buffer D containing 0.1 M NH_4Cl or until the A_{280} reading was nil. The IF-2 was eluted by washing the column with Buffer D containing 0.35 M NH_4Cl. The majority of the IF-2 activity was recovered in 1.0-1.5 ml (PC II IF-2). A 0.4 ml aliquot of the PC II IF-2 preparation was applied to a 50 cc Sephadex G-150 column equilibrated in Buffer D containing 1.0 M NH_4Cl, and the column was developed with the same buffer. Fractions of 0.5 ml were collected. The IF-2 activity emerged from the column at approximately 1.2 times the void volume. The peak frac-

tions were pooled; the volume was reduced 4-6 fold by dialysis against dry Sephadex G-100 to obtain a protein concentration of 0.15 to 0.2 mg per ml; and the G-150 IF-2 preparation was stored at -70°C. SDS gel electrophoresis showed one major and three minor bands with approximately 80% of the protein being in the major band of about 100,000 molecular weight. Proteins were determined by the Lowry method (11).

RESULTS AND DISCUSSION

As shown in Table 1, in the absence of IF-3 the amounts of fMet-tRNA

Table 1. Effects of IF-3 on the binding of fMet-tRNA and acPhe-tRNA to 30S and 70S ribosomes

Messenger	Ribosomes	fMet-tRNA or acPhe-tRNA Bound	
		Without IF-3	With IF-3
		pmoles	
AUG	30S	3.4	2.6
	70S	1.1	4.2
Poly (U,G)	30S	3.2	2.1
	70S	0.8	3.5
Poly (U)	30S	2.9	3.3
	70S	1.0	3.5
f_2RNA	30S	0.3	1.4
	70S	0.6	2.6
T_4late RNA	30S	0.4	1.4
	70S	0.4	3.4

The standard reaction mixture contained 1.9 μg of PC I IF-1, 1.6 μg of G-150 IF-2, and when indicated, 0.35 μg of PC II IF-3. Times and temperatures of incubation were as follows: 30S subunits and synthetic messengers, 5 minutes at 0°; 30S subunits and natural messengers, 10 minutes at 37°; 70S ribosomes and AUG or poly (U), 10 minutes at 25°; and 70S ribosomes and poly (U,G), f_2RNA or T_4late RNA, 10 minutes at 37°.

and acPhe-tRNA bound to isolated 30S subunits at the direction of synthetic messengers, AUG, poly (U,G) or poly (U), are 3 to 4 fold greater than the amounts bound to 70S ribosomes in the absence of IF-3. Addition of IF-3 enhances the binding of the initiator aminoacyl-tRNAs to 70S ribosomes but

does not enhance the binding to isolated 30S subunits directed by synthetic messengers. In fact, with AUG and poly (U,G), the amounts of fMet-tRNA bound to isolated 30S subunits are significantly and consistently lower in the presence of IF-3. This effect has been observed by other investigators (2,12). As would be expected, binding of fMet-tRNA to both 30S subunits and 70S ribosomes directed by natural messengers, f_2RNA and T_4 late RNA, is enhanced by the addition of IF-3. It should be noted that in contrast to previous reports (2,12), the amounts of fMet-tRNA and acPhe-tRNA bound to isolated 30S subunits with synthetic messengers in the absence of IF-3 are not appreciably lower than the amounts bound to 70S ribosomes in the presence of IF-3. Similarly, when the 30S subunits are incubated with synthetic messengers, initiator aminoacyl-tRNA, IF-2, IF-1, and GTP at 0°, and subsequently incubated with 50S subunits at elevated temperatures (25 or 37°), only a slight increase in binding is observed upon the addition of the 50S subunits (data not shown). Possible explanations for the near maximal binding obtained with 30S subunits and synthetic messengers are that the reaction mixtures contain close to stoichiometric amounts of IF-2 and 30S subunits, and are incubated at 0° rather than 25 or 37°.

The effects of temperature on the binding of fMet-tRNA to 30S and 70S ribosomes are shown in Table 2. With AUG or poly (U,G) less fMet-tRNA is bound to 30S subunits at 0° in the presence of IF-3 than in its absence. Also, less fMet-tRNA is bound at elevated temperatures (25-37°) either in the presence or absence of IF-3. In contrast, with f_2RNA very little binding of fMet-tRNA to 30S subunits is obtained at 0° with or without IF-3; enhanced binding is observed only at an elevated temperature in the presence of IF-3. Similarly, with 70S ribosomes and either f_2RNA, AUG or poly (U,G) very little binding of fMet-tRNA is obtained at 0° with or without IF-3, indicating that very little dissociation of 70S ribosomes occurs under these conditions. Maximal binding to 70S ribosomes is obtained only at elevated temperatures in the presence of IF-3. In separate experiments sucrose

Table 2. Effects of temperature on the binding of fMet-tRNA to 30S and 70S ribosomes

Messenger	Ribosomes	pmoles of fMet-tRNA Bound					
		Without IF-3			With IF-3		
		0°	25°	37°	0°	25°	37°
AUG	30S	3.4	2.3	—	1.9	2.0	—
	70S	0.2	1.5	—	0.3	4.2	—
Poly (U,G)	30S	3.2	—	2.2	2.1	—	2.0
	70S	0.2	—	0.8	0.2	—	3.0
f_2RNA	30S	0.4	—	0.2	0.4	—	1.4
	70S	0.1	—	0.3	0.1	—	2.7

The amounts of initiation factors in the reaction mixture were the same as for Table 1. Incubations were at the temperatures indicated above for the following times: 30S subunits and synthetic messengers, 5 minutes; 30S subunits and natural messenger, 10 minutes; 70S ribosomes and synthetic or natural messengers, 10 minutes.

Table 3. Effects of prior incubation of 70S ribosomes with IF-3 on the binding of fMet-tRNA directed by AUG and f_2RNA

Pretreatment of 70S Ribosomes		Subsequent Incubation	pmoles of fMet-tRNA Bound	
Additions	Temperature °C	Temperature °C	AUG	f_2RNA
IF-3	0	0	0.4	0.1
IF-3	0	37	4.2	2.6
IF-3	37	0	3.0	0.4
IF-3	37	37	—	2.5
—	37	0	—	0.1
—	37	37	—	0.2

The 70S ribosomes were incubated in standard reaction buffer (50 mM Tris HCl, pH 7.7, 5 mM DTT, 0.1 M NH_4Cl, and 5 mM $MgCl_2$) supplemented when indicated with 0.7 μg of PC II IF-3. After 5 minutes of incubation at the temperatures indicated above, the reaction mixture was supplemented with GTP, fMet-tRNA, AUG or f_2RNA, in the amounts given in Experimental, 1.9 μg of PCI IF-1 and 1.6 μg of G-150 IF-2. Subsequent incubation was for 10 minutes at the temperatures indicated above.

density gradient centrifugation of 70S ribosomes that had been incubated at 37° with stoichiometric amounts of IF-3 showed complete loss of the 70S peak with a corresponding increase in the size of the 30S and 50S subunit peaks.

The data given in Table 3 show that after incubation of the 70S ribosomes with IF-3 at 37°, AUG-directed binding of fMet-tRNA readily occurs at 0° but f_2RNA-directed binding does not. These data indicate that both of the IF-3 activities, i.e., its ability to enhance the dissociation of the ribosomes and its ability to enhance natural messenger RNA-directed binding of fMet-tRNA to the ribosomes, are greatly reduced at 0°.

Since binding of synthetic messengers to 30S subunits occurs in the absence of IF-3, we conclude that the IF-3 requirement observed in vitro with 70S ribosomes and synthetic messengers is not related to the ability of IF-3 to recognize specific nucleotide sequences, but rather can be attributed solely to the ability of IF-3 to enhance the dissociation of the ribosomes. The mechanism by which IF-3 recognizes different species of natural messengers or different initiation sites on a polycistronic messenger is still unclear (4, 13-16).

ACKNOWLEDGEMENTS

The authors are indebted to Dr. Severo Ochoa for making his manuscript available to us prior to publication. We are also indebted to Ms. Sandra Lax and Ms. Barbara Lade for their excellent technical assistance; and to Mr. Michael Haralson for the preparations of f_2RNA and T_4 late RNA; and to Ms. Janee Roche for the preparation of this manuscript. This work was supported in part by USPHS Grant BM 18775.

REFERENCES

1. Suttle, D. P., Haralson, M. A., and Ravel, J. M., Biochem. Biophys. Res. Comm. 51, 376 (1973).
2. Wahba, A. J., Iwasaki, K., Miller, M. J., Sabol, S., Sillero, M. A. G., and Vasquez, C., Cold Spring Harbor Symposium 34, 291 (1969).
3. Dufnoff, J. S., Lockwood, A. H., and Maitra, U., Arch. Biochem. Biophys. 149, 528 (1973).
4. Yoshida, M. and Rudland, P. S., J. Mol. Biol. 68, 465 (1972).
5. Miller, M. J. and Wahba, A. J., J. Biol. Chem. 248, 1084 (1973).
6. Bernal, S. D., Blumberg, B., Nakamoto, T., Fed. Proc. 32, 533 (1973).
7. Sabol, S., Sillero, M. A. G., Iwasaki, K., and Ochoa, S., Nature 228, 1269 (1970).

8. Subramanian, A. R. and Davis, B. D., Nature 228, 1273 (1970).
9. Ravel, J. M. and Shorey, R. L., Methods in Enzymology XX, 306 (1971).
10. Remold-O'Donnell, E. and Thach, R. E., Nature 222, 944 (1969).
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 256 (1951).
12. Meier, D., Lee-Huang, S., and Ochoa, S., J. Biol. Chem. 248, 8613 (1973).
13. Lee-Huang, S. and Ochoa, S., Biochem. and Biophys. Res. Comm. 49, 371 (1972).
14. Lee-Huang, S. and Ochoa, S., Arch. Biochem. Biophys. 156, 84 (1973).
15. Revel, M., Pollack, Y., Groner, Y., Scheps. R., Inovye, H., Berissi, H., and Zeller, H., Biochimie 55, 41 (1973).
16. Singer, R. E., and Conway, T. W., Biochim. Biophys. Acta 331, 102 (1973).