*POLYPEPTIDE CHAIN INITIATION IN E. COLI: STUDIES ON THE FUNCTION OF INITIATION FACTOR F_1**

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Communicated April 24, 1969

Abstract.—The requirement of initiation factors F_1 (highly purified) and F_2 (electrophoretically homogeneous) for ribosomal binding of N-formylmethionyl transfer RNA (fMet ~ tRNA) at low Mg^{2+} concentration (3.5 mM), with the trinucleoside diphosphate ApUpG as messenger, was studied under various experimental conditions with 30*S* + 50*S* ribosomes and with 30*S* subunits alone. The results were qualitatively the same in both cases but the amount of binding was two to three times higher when both 30*S* and 50*S* subunits were present.

Although there was a virtually absolute requirement for F_2 in all cases, considerable binding occurred at 0° in the absence of added F_1. F_1 addition stimulated binding up to twofold under these conditions. However, at 25°, the temperature at which the reaction is usually carried out, there was very little binding with F_2 alone and addition of F_1 stimulated the reaction five- to sixfold.

Contrary to current belief, the GTP analog 5′-guanylyldiphosphonate (GMP-PCP) cannot replace GTP in the binding reaction. In particular, there was but little stimulation of binding (about 1.5-fold) by addition of F_1 to F_2-containing samples when GMP-PCP was used. In marked contrast, binding was stimulated up to sevenfold by addition of F_1 when GTP was substituted for the analog. Under these conditions, there was an ApUpG and F_1-dependent hydrolysis of GTP. This is observable with 30*S* subunits alone and can hardly be related to the occurrence of translocation.

The results may be interpreted to mean that a complex relatively stable at 0°, but less stable at 25°, is formed upon addition of F_2 alone. Conversion of the less stable to the more stable form of complex is made possible by addition of F_1. This is accompanied or mediated by cleavage of GTP.

The role of the initiation factors in formation of the polypeptide chain initiation complex in *E. coli* has been the subject of many publications from various laboratories (for literature see bibliography of ref. 1). The complex can be formed on the 30*S* ribosomal subunit in the presence of messenger, fMet ~ tRNA, and Mg^{2+}. At low Mg^{2+} concentrations (3.5–5.0 mM) complex formation is strongly dependent on the additional presence of GTP and initiation factors. With the trinucleoside diphosphate ApUpG (AUG) as messenger, F_1 and F_2 are the main initiation factors required. With natural messengers, such as phage RNA, all three initiation factors, F_1, F_2, and F_3, must be present. The role of GTP is thought to be of a conformational nature, for the GTP analog 5′-guanylyldiphosphonate (GMP-PCP), which cannot be hydrolyzed, can reportedly substitute for GTP in initiation complex formation.

Most of the studies on chain initiation in our laboratory and others, were carried out with factors isolated according to our original procedure,[2] by ammo-

nium sulfate precipitation from the 1.0 *M* NH_4Cl ribosomal wash and fractionation by DEAE-cellulose chromatography. The individual factors in these rather crude preparations have varying degrees of contamination with each other and with inhibitors of chain initiation. Contamination may have been mainly responsible for the reported equal activity of F_1 and F_2 when used singly,[3] or the inhibitory effect of F_1 on 30*S* ribosomal binding of fMet $\sim$ tRNA in the presence of F_2.[4] We have recently succeeded in isolating F_2 as an electrophoretically homogeneous protein.[5] Highly purified preparations of F_1 have also been obtained.[6]

Using the highly purified factors we have studied the function of F_1 and F_2 in the AUG-dependent formation of the initiation complex with 30*S* and 30*S* + 50*S* ribosomal subunits, under various experimental conditions. The results with 30*S* and 30*S* + 50*S* ribosomes are qualitatively the same but the amount of binding is increased by the presence of the 50*S* subunits. With F_2 alone there is formation of an unstable complex which, on addition of F_1 is converted to a more stable form. This conversion requires GTP, which undergoes simultaneous hydrolysis. GMP-PCP cannot substitute for GTP.

Materials and Methods.—Purified *E. coli* Q13 ribosomes and ribosomal subunits were prepared as in previous work.[5, 7] Incubations were conducted essentially under the conditions of the standard F_2 assay,[5] except that fraction *S*, a crude ammonium sulfate fraction of the 1.0 *M* NH_4Cl ribosomal wash, was omitted and 5 mM dithiothreitol was included. The final concentrations of NH_4Cl in the assay were 170, 220, and 140 mM for the experiments of Tables 1–3, 4, and 5–6, respectively. All values given in the tables are averages of closely agreeing duplicate runs. Ribosomal binding of (^{14}C)Met $\sim$ tRNA was measured by the Millipore filter procedure as previously.[5, 7] Samples incubated at 0° were filtered in the cold room (4°). Contaminating GTP was removed from fMet $\sim$ tRNA preparations by gel filtration on Sephadex G-50.

Electrophoretically homogeneous F_2 (Step 6, F_2)[5] was used throughout. The preparation of F_1 used in all but one experiment was obtained[6] by chromatography on carboxymethyl cellulose (CMC) of DEAE-cellulose fractions prepared as previously described.[2] This step achieved on the average a 25-fold purification of the F_1 present in DEAE fractions. At this stage disc electrophoresis (pH 4.5, 8.0 *M* urea) showed a strong protein band along with some minor ones. This preparation was contaminated with a weak N-substituted aminoacyl $\sim$ tRNA hydrolyzing activity.[8] The F_1 preparation utilized in experiment 1 of Table 2 had been further purified by chromatography on phosphocellulose.[6] It gave essentially a single band on disc electrophoresis as above. Presence of contaminating activities was not assayed for. F_1 is a basic protein of molecular weight about 8000 as determined in a preliminary sedimentation equilibrium run by Dr. R. C. Warner. Availability of the more purified samples of F_1 was essential for this work because the DEAE fractions previously used were found to contain inhibitors of the binding reaction.

GTP hydrolysis was determined with use of GTP labeled with ^{32}P in the γ phosphate, as previously described,[5] except that the incubation mixtures had the same composition as in the binding assays and the concentration of GTP was 0.025 mM. An incubation with all components except ribosomes, F_2, and F_1 was utilized as a blank. Formylmethionyl puromycin (fMet-puro) synthesis was determined after incubation in standard binding assay reaction mixtures, by the procedure of Leder and Bursztyn[9] as previously described,[10] except that the pH of the samples was brought to 5.5 by addition of 1.0 ml of 0.1 *M* acetate buffer prior to the extraction of the fMet(^{14}C)-puro into ethylacetate.

Two batches of GMP-PCP were used. One was a gift of Dr. Lionel Simon, Northwestern University School of Medicine, Chicago; the other was purchased from the Miles Laboratories. Other preparations were as in previous work.[7]

Results.—Two groups of experiments were carried out: (*a*) with unfractionated ribosomes and (*b*) with ribosomal subunits. In the ionic environment of the binding assay (50 mM Tris-HCl buffer, pH 7.2, 150 mM NH_4Cl, 3.5 mM Mg^{2+}), the unfractionated ribosomes consist of a mixture of about 40 per cent 70*S* couples and 60 per cent 30*S* and 50*S* subunits.

(a) *Unfractionated ribosomes: Binding at 0° and 25°:* At 25°, the usual temperature in binding studies, binding is low with F_2 alone and addition of F_1 causes a large increase.[5] Surprisingly, when the reaction was carried out at 0° there was considerable binding with F_2 (Table 1). It was doubled by addition of F_1. At 25° binding with F_2 was marginal and was considerably increased by addition of F_1 (about sixfold). These results suggest that F_2 causes formation of a complex which, although relatively stable at 0°, is very unstable at higher temperatures. They further suggest that a more stable complex is formed when F_1 is also present. It should be noted that the amount of complex found at 25° with the complete system was less than at 0° without F_1, an indication that even in the presence of F_1 the complex is not stable at 25°.

TABLE 1. *AUG-dependent ribosomal binding of fMet~tRNA at 0° and 25°.*

Experiment no.	Temperature	Factor Additions (μg) F_2	F_1	fMet binding*	Stimulation by F_1
1	0°	0.2	None	4.6	
		"	1.0	8.4	×1.8
	25°	"	None	0.6	
		"	1.0	3.4	×5.7
2	0°	"	None	3.0	
		"	1.0	5.7	×1.9
	25°	"	None	0.4	
		"	1.0	2.2	×5.5

Conditions of standard F_2 assay with unfractionated ribosomes (2.5 A_{260} units). Incubation 15 min. Factors: Step 6 F_2; F_1, DEAE fraction further purified by CMC chromatography.

* Net values (blanks with no added factors subtracted) in micromicromoles/sample. The blanks (essentially the same in both experiments) were 0.8 at 0° and 0.2 at 25°.

Requirement of GTP for F_1 effect: Since GMP-PCP has been reported to substitute for GTP in formation of the chain initiation complex,[1] we carried out some experiments at 25° comparing each nucleotide in the absence and presence of F_1. The results (Table 2) show that GMP-PCP promoted significant binding in the absence of F_1. In fact, the amount of binding was about the same with either GMP-PCP or GTP when large amounts of F_2 (0.8 μg) were used. However, although F_1 caused considerable stimulation of binding with GTP, it was virtually inactive with GMP-PCP. Stimulation by F_1 in the presence of GTP ranged from about four- to sevenfold depending on the amount of F_2 present. The results clearly show that GMP-PCP can only replace GTP to a limited extent. It is tempting to assume that whereas GMP-PCP can replace GTP in the F_2-dependent formation of the less stable complex, presumably through a conformational effect, it cannot do so in the F_2- and F_1-dependent formation of the more stable one, the initiation complex proper.

The requirement of GTP for the F_1 effect suggested that there might be concomitant hydrolysis of the nucleoside triphosphate. Table 3, in which ribosomal binding of fMet~tRNA, fMet-puro synthesis, and GTP hydrolysis were assayed in parallel runs, shows that this was indeed the case.

TABLE 2. *GTP dependence of F_1 activity with 70S ribosomes.*

Experiment no.	F_2 (μg)	fMet(^{14}C)~tRNA Binding* With GMP-PCP: No F_1	With GMP-PCP: +F_1	With GMP-PCP: Stimulation by F_1	With GTP: No F_1	With GTP: +F_1	With GTP: Stimulation by F_1
1	0.2	0.39	0.63	×1.6	0.71	4.78	×6.7
	0.4	0.72	1.07	×1.5	1.41	8.03	×5.7
	0.8	1.83	2.14	×1.2	2.31	9.43	×4.1
2	0.4	0.79	1.14	×1.4	1.47	7.05	×4.8
	0.8	1.68	1.92	×1.1	1.83	8.68	×4.7

Conditions of Table 1 except that the preparation of F_1 used in experiment 1 had been purified by DEAE-cellulose, CMC, and phosphocellulose chromatography. Incubation, 15 min at 25°. The concentration of GMP-PCP or GTP was 0.2 mM.

* Net values in micromicromoles/sample as in Table 1. The blanks without factors, or with F_1 alone, were about 0.2 in all cases. Blanks with both factors but without GMP-PCP or GTP averaged 0.3.

TABLE 3. *Effect of F_1 on AUG-dependent ribosomal binding of fMet~tRNA, fMet-puromycin synthesis, and GTP hydrolysis.*

Factor additions	fMet binding	fMet-puro synthesis	GTP hydrolysis
F_2	0.76	1.92	8.6
F_1 + F_2	3.30	7.29	21.8
Increase due to F_1	2.54	5.37	13.2

Factor fractions and amounts as in Table 1. Standard assay for fMet binding. Conditions for fMet-puro synthesis and GTP hydrolysis as described in *Materials and Methods*. Unfractionated ribosomes, 2.5 A_{260} units. Incubation, 15 min at 25°. All values are given in micromicromoles/sample. Blanks without factors for fMet binding (0.2) and fMet-puro synthesis (0.8) were subtracted. In the case of GTP hydrolysis, the blank subtracted (32.2) was that given by samples containing ribosomes, fMet~tRNA, and F_2, but no AUG. This corrects for ribosome-dependent GTPase due to contamination of F_2 with the elongation factor *G*.[5]

(b) *Ribosomal subunits:* AUG- and initiation factor-dependent ribosomal binding of fMet~tRNA has been shown to occur with 30*S* subunits.[1] The question therefore arose whether the complex formed under these conditions is only F_2-dependent, in other words, whether the effect of F_1 requires the additional presence of 50*S* subunits. This was investigated with use of 30*S* and 30*S* + 50*S* ribosomes.

Binding at 0° and 25°: As shown in Table 4, 30*S* subunits behaved qualitatively in the same way as the combination of 30*S* and 50*S* ribosomes. There was good binding at 0°, but not at 25°, with F_2 alone. Addition of F_1 increased binding moderately (1.3-fold) at the former and markedly (fivefold) at the latter temperature (cf. Table 1). However, with both 30*S* and 50*S* subunits, binding was severalfold higher than with 30*S* subunits alone.

Requirement of GTP for F_1 effect: The above results show that the F_1-dependent conversion of the less stable to the more stable complex occurs with 30*S* ribosomes and does not require the presence of 50*S* subunits. We therefore carried out experiments similar to those of Table 2 with 30*S* ribosomes. As seen in Table 5, GMP-PCP could only partially substitute for GTP under these conditions, a result similar to those seen in Table 2. As shown in Table 6, F_1 caused a pronounced increase in GTP hydrolysis with 30*S* subunits.

Discussion.—In the work reported here we have examined the AUG-dependent ribosomal binding of fMet~tRNA at low Mg^{2+} concentration in regard to its requirements for F_1, F_2, and GTP, using highly purified F_1[6] and electrophoreti-

TABLE 4. *AUG-dependent binding of fMet∼tRNA with ribosomal subunits at 0° and 25°.*

Temperature	Subunits	Factor Additions (μg) F_2	F_1	fMet binding*	Stimulation by F_1
0°	30S	0.4	None	1.36	
		"	1.0	1.72	×1.3
	30S + 50S	"	None	4.0	
		"	1.0	8.0	×2.0
25°	30S	"	None	0.4	
		"	1.0	1.9	×4.7
	30S + 50S	"	None	0.68	
		"	1.0	4.76	×7.0

Conditions of Table 1, except that ribosomal subunits (30S, 0.8 A_{260} unit; 50S, 1.6 A_{260} units) were used. As assayed by polyU-dependent polyphenylalanine synthesis the contamination of 30S with 50S subunits was about 10%.

* Net values (blanks with no added factors subtracted) in micromicromoles/sample. The blanks at 0° were 0.25 and 0.5, respectively, for 30S and 30S + 50S subunits and, at 25°, 0.2 in both cases.

TABLE 5. *GTP-dependence of F_1 activity with 30S ribosomal subunits.*

Factor additions	fMet(^{14}C)∼tRNA Binding* With GMP-PCP	With GTP
F_2 (0.8 μg)	0.32	0.95
F_2 (0.8 μg) and F_1 (1.5 μg)	0.58	4.35
Stimulation by F_1	×1.8	×4.6

Conditions of Table 2 (experiment 2) but with 30S ribosomal subunits (0.9 A_{260} unit) only. As assayed by polyU-dependent polyphenylalanine synthesis, the contamination of 30S with 50S subunits was about 7%.

* Net values in micromicromoles/sample as in Table 1. The blanks without factors averaged 0.1. Blanks without GMP-PCP or GTP but with F_2 or F_2 + F_1 averaged 0.14 and 0.25, respectively.

cally homogeneous F_2.[5] Experiments were performed both with 30S + 50S ribosomes and with 30S subunits alone. We should like to emphasize that in the absence of added F_2, F_1 was devoid of activity. Binding or GTP hydrolysis with F_1 were about the same as with no factors.

Some new facts have emerged from this investigation. (1) Whereas addition of F_2 as the only factor promotes considerable binding at 0°, this is not the case at 25°. Supplementation with F_1 increases binding at 25° much more than it does at 0° (Table 1 and 4). This suggests that F_2 is sufficient to cause formation of an unstable AUG-ribosome-fMet∼tRNA complex and that F_1 is necessary for conversion of this complex to a more stable one. It should be noted, however, that even this complex is rather unstable at 25° (cf. Table 1). (2) GMP-PCP can replace GTP only partially for, although it increases binding to some extent at 25°, it does not permit the pronounced increase in binding seen in the presence of GTP when the system is supplemented with F_1 (Tables 2 and 5). (3) The increased binding is accompanied by GTP hydrolysis (Tables 3 and 6). The results suggest that F_1 is essential for formation of the initiation complex proper and that this process is coupled with GTP cleavage.

Coupled hydrolysis of GTP under similar conditions has recently been reported by Kolakofsky *et al.*[11] However, their interpretation that GTP hydrolysis reflects translocation of fMet∼tRNA from an aminoacyl (*A*) binding site on the 30S to a peptidyl (*P*) binding site on the 50S subunit is barely tenable in view of the fact that it occurs with 30S subunits alone (Table 6). It is not possible at this time to identify either F_2 or F_1 as directly involved in GTP cleavage. It

TABLE 6. *Effect of F_1 on AUG-dependent hydrolysis of GTP with 30S ribosomal subunits.*

Additions to system	GTP Hydrolysis* (a)	(b)
fMet + 30S + F_2 (0.8 μg)	18.8	—
fMet + 30S + F_2 (0.8 μg) + AUG	47.4	28.6
fMet + 30S + F_2 (0.8 μg) + AUG + F_1 (1.5 μg)	94.6	75.8
Increase due to F_1		47.2

General conditions of Table 5 with the same batch of 30S subunits (0.9 A_{260} unit). The GTP concentration was 0.025 mM. Incubation, 15 min at 25°. Other details as described in *Materials and Methods.*

* In column (b), the AUG-independent hydrolysis of GTP (18.8 $\mu\mu$moles) was subtracted.

appears to us rather that this cleavage is the result of a concerted, F_2-dependent interaction of fMet~tRNA with a site on the 30S ribosome of which F_1 may be a component. It has been shown[12, 13] that the initiation factors are associated with the 30S ribosomal subunits.

Under our conditions fMet~tRNA binding was always markedly increased when the 30S ribosomes were supplemented with 50S subunits, an effect which may be the result of increased stability of the complex in the presence of the latter. Qualitatively, however, the results were essentially the same with 30S or with 30S + 50S ribosomes. This means that the 50S subunits are not required for the activity of F_1 described here.

One final point that deserves some comment is the stoichiometry of complex formation with regard to F_2. Since the molecular weight of F_2 is approximately 80,000,[5] 1.0 $\mu\mu$mole of F_2 corresponds to about 0.08 μg. It may be seen from Table 1 that at 25°, in the presence of F_1, 0.2 μg (2.5 $\mu\mu$moles) of F_2 caused the binding of nearly stoichiometric amounts of fMet~tRNA (an average of 2.8 $\mu\mu$moles). However, the complex is clearly unstable at this temperature, for under the same conditions at 0° the same amount of F_2 promoted the binding of an average of 7 $\mu\mu$moles of fMet~tRNA or about 3 $\mu\mu$ moles/$\mu\mu$ mole F_2. This suggests that F_2 may undergo a cycle of association to and dissociation from ribosomes during initiation.

We wish to thank Miss Roswitha Pagel for her excellent technical assistance and Mr. Horace Lozina for growth of bacterial cells for preparation of ribosomes. We are much indebted to Dr. M. A. G. Sillero and Dr. A. J. Wahba for a supply of highly purified F_1.

* Aided by grants AM-01845, FR-05099, and GM-01234 from NIH.

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