*GUANOSINE 5'-TRIPHOSPHATASE ACTIVITY OF INITIATION FACTOR f_2**

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Communicated by Paul Doty, September 13, 1968

Guanosine 5'-triphosphate (GTP) is required in the binding of N-formylmethionyl-tRNA (F-met-tRNA) to ribosomes and for the formation of the first peptide bond.[1-5] 5'-Guanylylmethylenediphosphonate (GMPPCP), an analogue of GTP, can substitute for GTP in the binding reaction, but the F-met-tRNA bound in this manner cannot react with aminoacyl-tRNA or puromycin to form a dipeptide.[1, 3, 6, 7] These central facts have led to the development of the following model for the initiation of protein synthesis.[3, 4, 6-8] F-met-tRNA is first bound to the aminoacyl or A site on the ribosome (30*S*). GTP is required for this reaction but is not hydrolyzed. A 50*S* subunit is added, and the F-met-tRNA is then translocated to the peptidyl or P site where it can now react with an aminoacyl-tRNA. GTP is hydrolyzed in this translocation step. In the above scheme, initiation factors are also required,[9-11] but the nature of these factors and the way in which they participate in the above reactions remains obscure.

Recently, Erbe and Leder[12] have shown that G factor is not required for the formation of the first peptide bond but is required for the formation of the second and presumably for the subsequent peptide bonds. G factor is known to catalyze the GTP-dependent translocation of peptidyl-tRNA[12-14] during chain elongation and, according to the above model of initiation, F-met-tRNA must be similarly translocated. However, since G factor is not required in the formation of the first peptide bond, it seems likely that the translocation of F-met-tRNA in chain initiation is performed by one of the initiation factors. We have therefore investigated the initiation factors for GTPase activity and now report that f_2 catalyzes the ribosomal-dependent hydrolysis of GTP, and more importantly, most of this activity is dependent upon the binding of F-met-tRNA to ribosomes.

Materials and Methods.—Ribosomes, ApUpG, and unfractionated F-met-tRNA were all prepared as previously described.[3, 4] Initiation factors were extensively purified by methods to be described in detail elsewhere.[15] The preparations of f_1 and f_2 used in this study were more than 95% and 30% pure, respectively, as estimated from polyacrylamide gel electrophoresis.

Met-tRNA$_f$ and met-tRNA$_m$ were prepared by countercurrent distribution.[16] Charging and formylation were carried out as previously described,[3, 4] and base-hydrolyzed aliquots were analyzed by paper chromatography as a test of purity. The preparations of F-met-tRNA$_f$ and met-tRNA$_m$ were not cross-contaminated, as judged by this test.

GTP-γ-P^{32} of original specific activity 14.3 c/mm was purchased from International Chemical and Nuclear Corp., and diluted with cold GTP before use.

Unless otherwise noted, standard reaction mixtures contained, in a total volume of 25 μl: 50 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl), pH 7.4, 100 mM NH_4Cl, 10 mM Mg acetate, 10 mM 2-mercaptoethanol, 50 μg of ribosomes, and 0.02 mM GTP-γ-P^{32} (from 40 to 200 cpm per $\mu\mu$mole). Where indicated, 0.2 μg of f_1, 1.2 μg of f_2, 1.0 μg of AUG, and 16 μg of tRNA charged with methionine in the presence of a formyl donor were added. Reactions were started by the addition of GTP and were continued

for 30 min at 30°C. Reactions were stopped by adding 0.1 ml of 1 mM potassium phosphate buffer, pH 7.0; the tubes were then placed in dry ice for storage. The reaction mixtures were thawed, and 0.1 ml of 1 *M* $HClO_4$, followed by 1.0 ml of potassium phosphate buffer, pH 7.0, and 0.5 ml of 5% ammonium molybdate in 4 *N* H_2SO_4, were added. The phosphomolybdate complex formed was extracted by vigorously shaking the solution with 2.0 ml of an equal mixture of isobutanol and benzene. 1.6 ml of the organic phase was then removed and added to 10 ml of toluene scintillation fluid containing 0.1 ml of hydroxide of hyamine (Packard Instrument Co.) and counted.

Results.—Using the standard assay described above, we first sought to determine whether either of the initiation factors f_1 or f_2 exhibited GTPase activity. As can be seen in Figure 1, f_2 shows little activity by itself but is strongly stimulated by ribosomes. Thus, like G factor,[13, 14] f_2 is a ribosome-dependent GTPase. In contrast, f_1 is completely inactive both by itself and in the presence of ribosomes (Table 1).

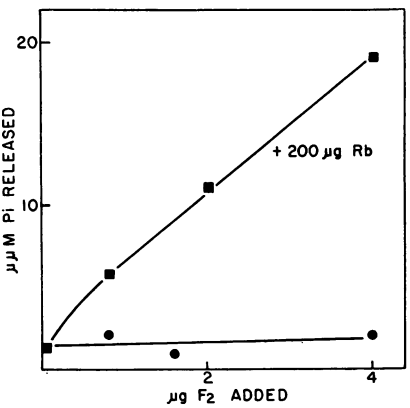

FIG. 1.—The effect of ribosomes on the GTPase activity of f_2. Reaction mixtures were as described in *Materials and Methods*, except that the final volume was 100 μl and incubation was for 10 min at 30°C.

Experiments were then conducted to see whether the GTPase activity of f_2 could be further stimulated by the other components required for normal initiation, namely, f_1, ApUpG, and F-met-tRNA. The results of these experiments are shown in Table 1. Note that f_1 shows no activity in the presence or absence of ApUpG and F-met-tRNA. As previously demonstrated, f_2 exhibits GTPase activity in the presence of ribosomes, but is also stimulated somewhat by the addition of ApUpG and F-met-tRNA. This latter stimulation may be correlated with the fact that there is a low level of binding of F-met-tRNA to ribosomes with f_2 alone. Nevertheless, as is the case with binding, f_1 strongly enhances the activity observed with f_2. Thus, the maximal level of GTPase activity is obtained only in the presence of all components normally required for the binding reaction. This suggests that the binding of F-met-tRNA to the ribosomes is a necessary prerequisite to the GTPase reaction. It becomes helpful at this point to define the level of GTPase activity obtained with ribosomes and f_2 alone as the

TABLE 1. *The effect of AUG and F-met-tRNA on the GTPase activity of f_1 and f_2.*

Additions	μμM Pi Released			
	Expt. 1		Expt. 2	
	Total	Δ	Total	Δ
Ribosomes	10.0		4.0	
Ribosomes + f_1	9.6	<0	3.9	<0
Ribosomes + f_1 + ApUpG + F-met-tRNA	7.4	<0	3.5	<0
Ribosomes + f_2	22.2	12.2	13.7	9.8
Ribosomes + f_2 + ApUpG + F-met-tRNA	27.3	17.3	17.0	13.1
Ribosomes + f_1 + f_2 + ApUpG + F-met-tRNA	41.3	31.3	27.3	23.4

Reaction conditions are described in *Materials and Methods*.

"uncoupled" activity. Stimulation above this level shall be called "coupled," because this increase in activity seems dependent on, or coupled to, the binding of F-met-tRNA to ribosomes.

To further demonstrate that this enhancement of f_2's GTPase activity is due to the formation of the initiation complex (F-met-tRNA, ApUpG, ribosome, and initiation factors), the kinetics of this reaction were studied (Fig. 2). This experiment clearly shows that the maximal rate of GTP hydrolysis is obtained

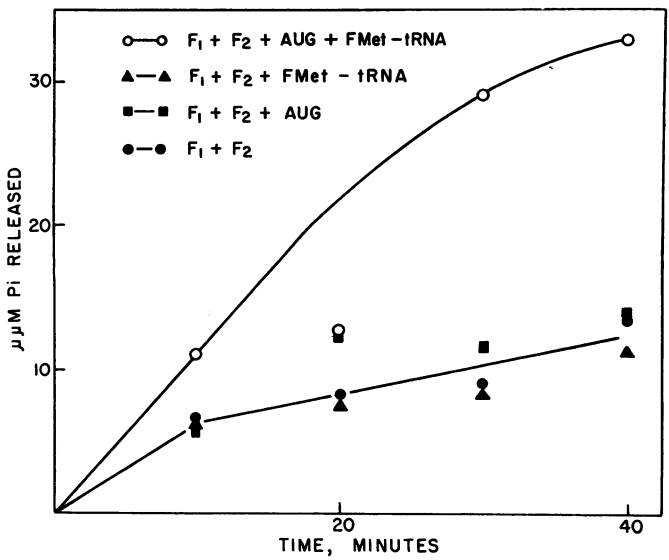

FIG. 2.—The effect of ApUpG and F-met-tRNA on the GTPase activity of initiation factors. Reaction conditions were as described in *Materials and Methods*, except that incubation was carried out at 25°C.

only in the presence of all components required for optimal binding. Further studies on the specificity of this reaction (not shown here) indicate that neither poly U nor uncharged tRNA can replace ApUpG or F-met-tRNA, respectively.

Because the F-met-tRNA used in the above experiments was prepared from unfractionated tRNA in the usual manner,[3, 4, 6] it contained not only F-met-$tRNA_f$, but also met-$tRNA_m$ and possibly met-$tRNA_f$. To determine whether the coupled GTPase activity of f_2 was due to a particular one of these species, and if so, which one, all three possible met-tRNA's were tested. The results are reported in Table 2. Although both met-$tRNA_m$ and met-$tRNA_f$ slightly inhibit the reaction, F-met-$tRNA_f$ exhibits strong stimulation similar to unfractionated F-met-tRNA. Thus it is clear that the formyl group of F-met-$tRNA_f$ is required for the coupled GTPase activity of f_2.

The preparation of f_2 used in these experiments, although highly purified, was not homogenous. Thus, the possibility remained that this preparation of f_2 contained G factor and that the GTPase activity observed was due to this contamination. This would be possible if f_2 were involved only in the preliminary

TABLE 2. *Specificity of the met-tRNA responsible for the coupled GTPase activity of f_2.*

Additions	$\mu\mu$M P_i Released	
	Total	Δ
Ribosomes	10.3	
Ribosomes + f_1 + f_2	25.9	15.6
Ribosomes + f_1 + f_2 + ApUpG + met-tRNA$_f$	19.3	9.0
Ribosomes + f_1 + f_2 + ApUpG + met-tRNA$_m$	20.4	10.1
Ribosomes + f_1 + f_2 + ApUpG + F-met-tRNA$_f$	39.9	29.6
Ribosomes + f_1 + f_2 + ApUpG + F-met-tRNA*	40.6	30.3

The reaction conditions were the same as described in *Materials and Methods*, except that 14.2 μg of met-tRNA$_f$, 13.2 μg of met-tRNA$_m$, 10.8 μg of F-met-tRNA$_f$, and 16.0 μg of F-met-tRNA* were used.

* Unfractionated.

binding of F-met-tRNA to ribosomes, whereas the contaminating G factor were responsible for its subsequent translocation and GTP hydrolysis. To rule out this possibility, a preparation of G factor (the kind gift of Dr. Nathan Brot) and the preparation of f_2 were compared for heat stability. The heat inactivation curves are shown in Figure 3. It is clear that the uncoupled GTPase activity of f_2 is one third as stable as that of G factor at 60°C and therefore cannot be due to contaminating G factor.

Under the conditions of the GTPase assay described above, binding of F-met-tRNA to ribosomes takes place quite readily and is approximately 30 per cent efficient with respect to ribosomes. While this binding is completed within 10 to 15 minutes (data not shown), the kinetics of GTPase activity are linear for at least 30 minutes. Nevertheless, within the initial ten minutes of the reaction

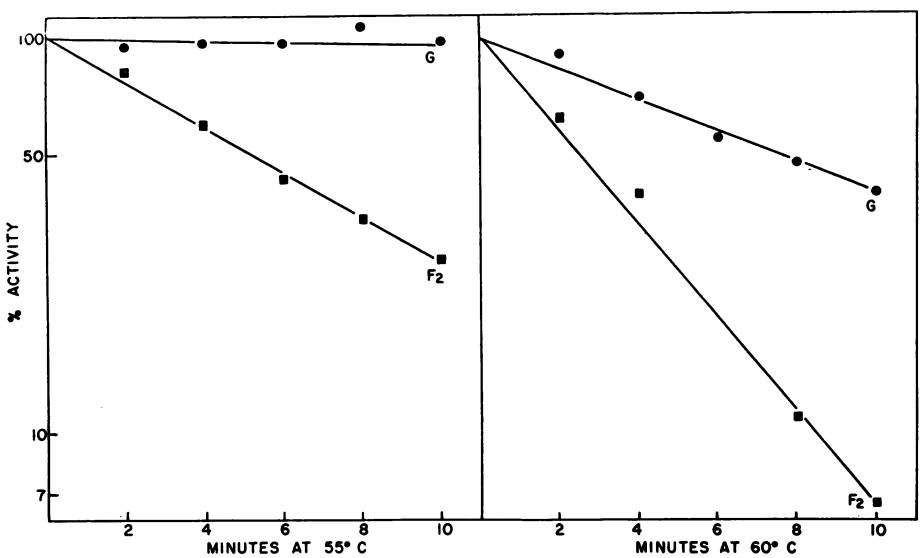

FIG. 3.—The effect of temperature on the uncoupled GTPase activities of f_2 and G factor. Both f_2 and G factor were heated simultaneously in a solution containing 20 mM Tris-Cl, pH 7.4, 0.3 *M* NH_4Cl, 1.0 mM DTT, and 15% glycerol, for various times at 55° and 60°C. GTPase activity was determined as previously described, except that 2.0 μg of f_2 or 0.2 μg of G factor were used in each assay.

(see Fig. 2), a one-to-one molar ratio exists between GTP hydrolysis and F-met-tRNA binding to ribosomes (the uncoupled level of GTPase activity is taken as background).

Discussion.—The evidence just presented demonstrates that at least one of the properties of f_2 is the catalysis of GTP hydrolysis dependent upon the binding of F-met-tRNA to ribosomes. F-met-tRNA bound to ribosomes in the presence of GTP is known to react with aminoacyl-tRNA or puromycin[3] and therefore, by definition, is in the P site. Inasmuch as recent evidence indicates that F-met-tRNA is first bound to the A site on the ribosome,[6-8] it is clear that a translocation step is required. Thus, initiation factors not only play a role in the initial binding of F-met-tRNA to ribosomes, but must also be involved in its subsequent translocation so that the first peptide bond can be formed. We therefore conclude that f_2 is the translocase responsible for this step.

Summary.—Initiation factor f_2 catalyzes the GTP-dependent translocation of F-met-tRNA from the A to the P site on the ribosome.

We are grateful to Paul Doty for advice, encouragement, and support. We also thank Edward Klem for the preparation of fractionated tRNA, Nathan Brot for his kind gift of G factor, and Olke Uhlenbeck, Frank Overlan, and Grace A. Porges for help in preparing the manuscript.

* This work was supported by grants from the National Science Foundation (GB-6109) and the National Institutes of Health (HD-01229).

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