THE ROLE OF GUANOSINE 5'-TRIPHOSPHATE IN THE INITIATION OF PEPTIDE SYNTHESIS, III. BINDING OF FORMYLMETHIONYL-TRNA TO RIBOSOMES*

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Recently we have demonstrated that guanosine 5'-triphosphate (GTP) is required for the formation of the first peptide bond in protein synthesis by an *E. coli* cell-free system.[1, 2] A preliminary investigation of the role of GTP in this reaction indicated that the GTP-dependent step occurred after the binding of formylmethionyl-tRNA (F-met-tRNA) to the ribosome. This conclusion was based on experiments which showed only a relatively small stimulation by GTP of the rate of the binding reaction.[1] However, a thorough investigation of this point has revealed a more complicated situation; it is now apparent that the effect of GTP on the binding reaction may vary markedly, depending upon the Mg^{++} concentration and the nature of the ribosome preparation used (that is, the relative proportions of 70*S*, 50*S*, and 30*S* subunits). Under certain conditions GTP is absolutely required for binding, whereas under others it merely stimulates the rate of the binding reaction. Our result agrees with recent reports from other research groups.[3–7] In addition we have observed that guanylyl-5'-methylene diphosphonate (GMP-PCP), a competitive inhibitor of peptide synthesis,[1, 2, 8] also stimulates the binding reaction.[6] The F-met-tRNA bound in the presence of GMP-PCP is unreactive with puromycin, whereas that bound with GTP reacts completely. This result suggests that the role of GTP in initiation may be complex, involving a series of distinct steps. The first is thought to be a binding of F-met-tRNA to a ribosome; the second is an activation step which renders the bound F-met-tRNA reactive to puromycin or a second aminoacyl-tRNA. The fact that GMP-PCP cannot substitute for GTP in the second step suggests that hydrolysis of the β-γ anhydride bond is involved, whereas this is probably not the case in the first step.

Methods.—All preparative methods were similar to those previously reported,[1] except for the washing of ribosomes and the preparation of initiation factors. Ribosomes were pelleted from an S-30 extract (*E. coli* strain 1113) and resuspended and stored overnight in Buffer A [10 mM Tris-HCl, pH 7.8, 40 mM $Mg(OAc)_2$, 10 mM β-mercaptoethanol, 2 mM EDTA, and 1 *M* NH_4Cl]. Ribosomes were pelleted from this solution by centrifugation for 6 hr at 105,000 × *g* (the supernatant is saved as a source of initiation factors), resuspended in Buffer A, clarified by low-speed centrifugation, and then repelleted. Ribosomes were resuspended in Buffer B [10 mM Tris-HCl, pH 7.8, 10 mM $Mg(OAc)_2$, 6 mM β-mercaptoethanol, and 50 mM NH_4Cl], clarified, and repelleted. This final pellet was resuspended in Buffer B, clarified, and stored at 0°. Ribosomes prepared by this method are virtually free of initiation factors; they consist of about 90% 70*S* ribosomes and 10% 50*S* subunit. There are no detectable 30*S* subunits present (see Table 1).

Initiation factors were prepared from the first ribosomal wash by filtration (Millipore filter, type HAWP) and precipitation with ammonium sulfate (65% saturated). The precipitate was redissolved in Buffer C (50 mM Tris-HCl, pH 7.8, 10 mM β-mercaptoethanol) and passed over a G-25 sephadex column equilibrated in the same buffer. The breakthrough peak of the eluate was charged onto a DEAE-cellulose column equilibrated with Buffer C containing 0.25 *M* NH_4Cl, and eluted with the same buffer. Protein was precipitated from the eluate with ammonium sulfate (80% saturated). The initiation factors prepared by this technique are virtually free of

contaminating RNA (the crude ribosomal wash usually contains sRNA, mRNA, and 30S ribosomes).

Binding assays were conducted essentially as described.[1] Details of reaction conditions are given in the legends of figures and tables. Simultaneous assay of puromycin sensitivity was performed by adding 30 μg of puromycin (in 3 μl) to a 30-μl aliquot of the binding reaction, incubating 3 min at 20°, and assaying the formylmethionyl-puromycin (F-met-puro) formed by the method of Leder and Bursztyn.[9]

Results.—Effect of preincubating ribosomes at low Mg^{++} concentration: The preparation of ribosomes and initiation factors described above (*Methods*) differs from that previously used[1] chiefly in that a higher salt concentration in the ribosomal wash buffer was employed (to ensure complete removal of factors), and in the use of the DEAE-cellulose step in the factor preparation. These procedural differences resulted in a system which displayed characteristics rather different from those previously observed. In the first place, the optimum Mg^{++} concentration for the new system is 4–5 mM, whereas previously it had been 8–10.[1] Second, the stimulation of the binding reaction by GTP is much more pronounced. In the process of optimizing conditions for the new system, it was found that preincubation of the ribosomes and factors at a very low Mg^{++} concentration (0.3 mM) restored several of the properties of the old system. Thus with preincubated ribosomes the optimum Mg^{++} concentrations for binding (as well as for F-met-puro synthesis) is around 10 mM Mg^{++}, and considerable binding is observed in the absence of GTP. This effect of preincubation at low Mg^{++} concentration is shown in Figure 1. In Figure 1*a*, the ribosomes and factors were preincubated together, but at the same Mg^{++} concentrations used during the subsequent binding reaction (from 5 to 14 mM); in Figure 1*b*, preincubation was at 0.3 mM Mg^{++}, whereas the concentration in the binding reaction was varied as indicated on the abscissa.

The optimum Mg^{++} concentration for the preincubation effect (i.e., a shift in the optimum Mg^{++} concentration for the binding step) was determined by varying the concentration during this step over a wide range, but raising it to a constant level (8 mM) for the subsequent binding step. This experiment revealed little effect of preincubation at Mg^{++} concentrations above 3 mM; however, as the concentration was reduced below this level there was a sharp increase in binding and F-met-puro synthesis, which was maximized at about 0.5 mM Mg^{++}. This

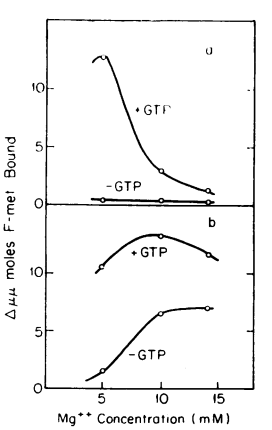

FIG. 1.—Effect of preincubation at low Mg^{++} concentration on binding of F-met-tRNA to ribosomes. Reactions were conducted in two stages, the first consisting of a preincubation of ribosomes (90 μg) and factors (57 μg) at various $Mg(OAc)_2$ concentrations for 10 minutes at 0°. The second stage, or binding reaction *per se*, was initiated by the addition of aminoacyl-tRNA (44 μg, charged with 0.1 mμmoles H^3-met, 19 cold amino acids, and cold formate), variable amounts of $Mg(OAc)_2$, and, as indicated, ApUpG (5.4 μg) and GTP (50 mμmoles), the final reaction volume being 50 μl. Tris-HCl buffer (pH 7.1, 0.05 *M*) and NH_4Cl (0.1 *M*) were present in both stages. Mixtures were incubated 10 min at 20° and assayed as previously described.[1] (*a*) $Mg(OAc)_2$ concentrations were maintained at the indicated levels in both stages of the reaction. (*b*) $Mg(OAc)_2$ concentration was 0.33 mM during preincubation, then raised to the indicated levels during the binding reaction. Background levels obtained from messenger blanks have been subtracted from all values. These varied from 1 to 3 $\mu\mu$moles in the presence of GTP, and from 0.3 to 0.9 $\mu\mu$moles in the absence of GTP.

result suggested that the preincubation effect corresponded to the dissociation of 70*S* ribosomes into 50*S* and 30*S* subunits, since both reactions are very similar in Mg^{++} concentration dependence.[10] To test this possibility, ribosomes were preincubated under varying conditions and analyzed on sucrose gradients. The degree of dissociation of 70*S* into 50*S* and 30*S* subunits is shown in Table 1. It is clear that at 10 mM Mg^{++} there is no detectable dissociation, whereas at 0.3 mM Mg^{++} dissociation is complete. At 5 mM Mg^{++}, the optimum for the system in which the concentration is held constant throughout the experiment (Fig. 2), 70*S* ribosomes are just beginning to dissociate.

TABLE 1

Mg^{++} concentration in ribosome sample and gradient	Fraction of 70*S* Dissociated into 50*S* and 30*S*	
	Experimental	Published value[10]
10 mM	<0.05	0
5 mM	0.13	0.14
0.33 mM	>0.95	1.00

The Mg^{++} concentrations of ribosome sample solutions and sucrose gradients are shown in the first column; in addition they contained 0.1 *M* NH_4Cl and 0.01 *M* Tris-HCl, pH 7.4. Centrifugation was for 6 hr at 39,000 rpm in a Spinco SW-39 rotor at 0°. The mole fraction of 30*S* subunits present was taken as a measure of the degree of dissociation of 70*S* ribosomes. The undissociated 70*S* preparation, as measured at 10 mM Mg^{++}, contained a 10% contaminant of 50*S* subunits (but no detectable 30*S*).

These results suggest that the effect of preincubation on the final Mg^{++} concentration optimum, and on the degree of GTP-dependence for binding, may be directly attributable to dissociation of 70*S* ribosomes into 50*S* and 30*S* subunits. If this is the case, it implies that the optimum Mg^{++} concentration for binding in the presence of a mixture of 50*S* and 30*S* subunits is 10 mM, whereas that for 70*S* ribosomes is 5 mM. (Recent experiments conducted with "native" 70*S*, 50*S*, and 30*S* ribosomes confirm this conclusion.) Since binding to 70*S* ribosomes is thought to be a simpler process than binding in presence of 50*S* and 30*S* subunits, initial experiments were conducted primarily with the non-preincubated (undissociated) system. Results with the preincubated (dissociated) system, as well as with isolated 50*S* and 30*S* ribosomes, will be reported elsewhere.

The finding that some of the properties of the old system[1] could be restored by dissociating 70*S* ribosomes into 50*S* and 30*S* subunits suggested that the preparative techniques previously used resulted in a heterogeneous ribosome population. This has recently been confirmed by an analysis of the ribosomal wash fluid previously used as a source of initiation factors. This wash contains variable amounts of 50*S* and 30*S* subunits. Thus it seems possible that these components played a major role in determining the properties of the old system. Whether or not these properties have biological significance remains to be determined; the results of Mangiarotti and Schlessinger[11] suggest that they may.

It is of interest to note that the Mg^{++} concentration dependence of the binding reaction recently reported by Leder and Nau[7] is very similar to that of the dissociated system, shown in Figure 1*b*.

Effect of GTP and GMP-PCP on binding F-met-sRNA to undissociated ribosomes: As noted above (Fig. 1*a* and Fig. 2), GTP is required for binding in the non-preincubated system, and the optimum Mg^{++} concentration for this binding is 5 mM. Surprisingly, GMP-PCP also stimulates binding of F-met-tRNA, as shown in Figure 2. It is about half as effective as GTP in this system, and the optimum Mg^{++} concentration is also 5 mM. Since GMP-PCP had been previously shown

to inhibit F-met-puro synthesis,[1] it seemed likely that the F-met-tRNA bound in its presence would not react with puromycin. That this is indeed the case is shown in Figure 3. All the F-met-tRNA bound in the presence of GTP reacts with puromycin,[12] while that bound with GMP-PCP is unreactive.

It should be noted here that similar experiments have been conducted with the preincubated (dissociated) system, with essentially the same result. However, as pointed out above, this system is complicated by the presence of a binding reaction which takes place in the absence of GTP or GMP-PCP. These experiments will be described elsewhere.

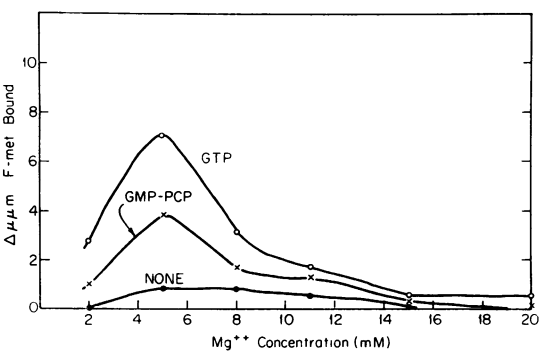

FIG. 2.—Binding of F-met-sRNA to ribosomes stimulated by GTP or GMP-PCP as a function of Mg^{++} concentration (no preincubation at low Mg^{++} concentration). Reaction mixtures contained 60 μg ribosomes, 45 μg factors, 33 μg aminoacyl-tRNA, 50 mμ moles GMP-PCP where indicated, and other components as described in Fig. 1. Ribosomes and factors were not preincubated as such, but the order of mixing of reaction components was regulated so that the $Mg(OAc)_2$ concentration never fell below 0.8 of the final level shown. Values have been corrected for messenger blanks, which were 0.5 to 1.5 $\mu\mu$moles with GTP and 0.2 to 0.4 with GMP-PCP and no addition.

The results in Figures 2 and 3 imply that the role of GTP in initiation may be complex, consisting of at least two separate but consecutive stages. The first stage would be the formation of an active complex composed of ribosome, F-met-tRNA, messenger, GTP, initiation factor(s), and perhaps supernatant factors. In promoting the formation of this complex GTP is presumably not hydrolyzed, since GMP-PCP can serve as a substitute. The F-met-tRNA bound in this form is not reactive with puromycin. This first complex is presumably transformed into a second type of complex by the hydrolysis of GTP to GDP and Pi.[13] In this new complex the F-met-tRNA remains bound to the ribosome, but is now reactive with puromycin. This transition from stage one to stage two can be accomplished by GTP, but not by GMP-PCP. The unreactivity of the latter may be ascribed to the resistance of the $P\text{-}CH_2\text{-}P$ linkage to hydrolysis, and is thought to account for the inhibitory effect of this compound on polypeptide synthesis.[8]

The binding of val-tRNA directed by $ApU(pG)_2\ (pU)_{\overline{25}}$: The roles of GTP and GMP-PCP in the initiation reaction have been further investigated in experiments which measure the reading of the second triplet in the message, that is, the triplet

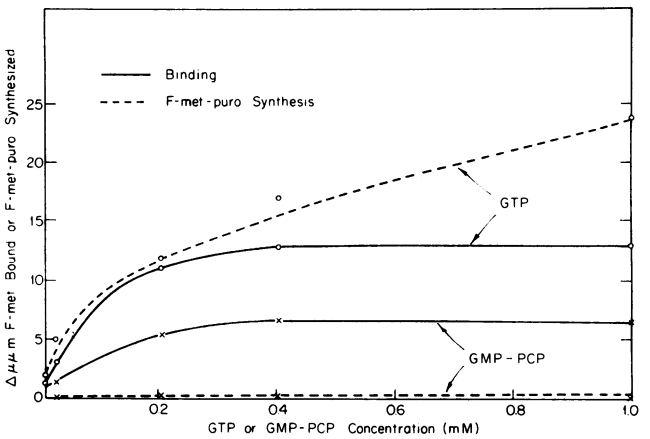

FIG. 3.—Binding of F-met-sRNA to ribosomes and synthesis of F-met-puro stimulated by GTP and GMP-PCP. Reaction conditions were as described in Fig. 2 and *Methods*. The Mg-$(OAc)_2$ concentration was 5 mM. Values have been corrected for messenger blanks, which were of the same order as in Fig. 2.

adjacent to the 3′ end of the ApUpG. . . sequence. The binding of val-tRNA directed by the messenger $ApU(pG)_2(pU)_{\overline{25}}$ has been studied under a wide variety of conditions, with and without the addition of GTP or GMP-PCP. A detailed account of these experiments will be presented elsewhere; however, results representative of those obtained under all conditions are shown in Table 2. It is evident that GMP-PCP is about half as effective as GTP in stimulating the binding of F-met-tRNA, as was observed with the triplet messenger ApUpG (Fig. 2). In contrast, binding of val-tRNA, which is directed by the second triplet in the message. . . GpUpU. . . , is observed only in the presence of GTP. GMP-PCP is totally inactive in this regard. These results again indicate that GMP-PCP is able to accomplish only the first of a series of functions normally performed by GTP. It can stimulate the binding of F-met-tRNA, but it cannot undergo a subsequent reaction which both renders the bound F-met-tRNA sensitive to puromycin, and, as shown by the present experiments, allows the binding of the second aminoacyl-tRNA.

TABLE 2

BINDING OF F-MET- AND VAL-tRNA DIRECTED BY $ApU(pG)_2(pU)_{25}$

Additions	F-met-tRNA bound (Δ μμmoles)*		Val-tRNA Bound (Δ μμmoles)*	
None	0.47	(0.44)	0.40	(0.40)
GMP-PCP	3.21	(0.87)	0.05	(0.40)
GTP	6.69	(1.57)	4.78	(0.67)

Reaction conditions were essentially as described in Figure 1*b*, except that the preincubation mixture contained 41 μg of supernatant fraction (S-100, freed of RNA by passage over a DEAE-cellulose column) and 1 mM GTP or GMP-PCP, where indicated (experiments have shown that GTP and GMP-PCP can be added either during preincubation or binding, with little difference in the result). After preincubation at 0.33 mM Mg^{++} at 0° for 10 minutes, the following were added to give a final volume of 50 μl: aminoacyl-sRNA (27 μg, charged with H^3-met, C^{14}-val, 18 cold amino acids, and cold formate), $ApU(pG)_2(pU)_{25}$ (5.2 μg), and $Mg(OAc)_2$ to give a final concentration of 5 mM.

* Figures in parentheses represent values obtained with reaction tubes in which the messenger was omitted.

Virtually all of the C^{14}-val bound in the presence of GTP is incorporated in oligopeptide, since the addition of puromycin renders all of the radioactivity soluble in ethyl acetate.[9]

Discussion.—In the present communication, a sufficient number of parameters of the binding reaction have been investigated to reveal that this is a complicated process. Of particular importance is the state of the ribosomes employed. Dissociation of 70*S* ribosomes into 50*S* and 30*S* subunits results in marked changes in the properties of the system, as evidenced by differences in optimum Mg^{++} concentration and degree of dependence on GTP. This observation seems important inasmuch as it may account for the considerable variation in Mg^{++} concentration optima reported for initiation in *E. coli* cell-free systems with F-met-tRNA or acetyl-phe-tRNA.[1, 3–7, 14–17] Thus it seems possible that the Mg^{++} concentration optimum observed in any particular case will reflect to a large extent the relative frequencies of the three classes of ribosomal particles in a given preparation. In this connection it is of interest to point out that in crude S-30 extracts, which contain a large proportion of 30*S* and 50*S* subunits,[11] the optimum Mg^{++} concentration for initiation with F-met-tRNA is 8–10 mM, as measured by a wide variety of techniques.[15–18] The relevance of this observation to the recent suggestion of Mangiarotti and Schlessinger[11] that initiation *in vivo* occurs not with 70*S* ribosomes but with 50*S* and 30*S* subunits is unclear. However, it is of interest to note that when 70*S* ribosomes are employed, optimum binding of F-met-tRNA occurs at a Mg^{++} concentration (5 mM) where dissociation into subunits is beginning to occur (Table 1).

In spite of the uncertainty as to whether *in vivo* initiation occurs with 70*S* ribosomes or with 50*S* and 30*S* particles, it is clear that the role of GTP in this reaction is complex. Experiments presented above show that this is the case for 70*S* ribosomes (undissociated system); that this is also generally true for the 50*S* plus 30*S* (dissociated) system will be documented elsewhere. The precise role of GTP remains to be illuminated, although the results presented above suggest that it may serve in at least two sequential functions. In the first of these, which involves the initial binding of F-met-tRNA to the ribosome, GTP is presumably not hydrolyzed. Hydrolysis probably occurs during the transition from the first to a second stage. In this second stage the bound F-met-tRNA is reactive with puromycin (whereas previously it was not), and the adjacent triplet in the message can direct the binding of the appropriate aminoacyl-tRNA. Whether the transition from stage one to stage two involves a "frame shift" mechanism[1, 19] or some other type of chemical activation[1] remains to be determined.

Summary.—Under certain conditions GTP or GMP-PCP are absolutely required for the binding of F-met-tRNA to ribosomes, whereas under other conditions they merely stimulate the rate of this reaction. F-met-tRNA bound in the presence of GMP-PCP does not react with puromycin; GMP-PCP promotes the reading of the first triplet in the message (ApUpG. . .), but not the second. These results suggest that the role of GTP in initiation may involve two or more sequential stages.

Note added in proof: Recently, Nomura and Lowry[20] have reported that 70*S* ribosomes do not bind F-met-tRNA in response to f2 viral RNA. Inasmuch as these experiments were performed at a relatively high Mg^{++} concentration (9–12 mM), the result is not inconsistent with our experiments using ApUpG as messenger (Fig. 1). If these authors had employed a lower Mg^{++} concentration (5 mM), our experiments suggest that they would have observed F-met-tRNA binding with 70*S* ribosomes. That this activity of 70*S* ribosomes at low Mg^{++} concen-

tration may be directly attributable to dissociation into 50*S* and 30*S* subunits remains a likely possibility.

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[1] Hershey, J. W. B., and R. E. Thach, these PROCEEDINGS, **57**, 759 (1967).

[2] Thach, R. E., K. F. Dewey, and N. Mykolajewycz, these PROCEEDINGS, **57**, 1103 (1967).

[3] Lucas-Lenard, J., and F. Lipmann, these PROCEEDINGS, **57**, 1050 (1967).

[4] Allende, J. E., and H. Weissbach, *Biochem. Biophys. Res. Commun.*, **28**, 82 (1967).

[5] Wahba, A. J., personal communication.

[6] Anderson, J. S., M. S. Bretscher, B. F. C. Clark, and K. A. Marcker, *Nature*, **215**, 490 (1967).

[7] Leder, P., and M. M. Nau, these PROCEEDINGS, **58**, 774 (1967).

[8] Hershey, J. W. B., and R. E. Monro, *J. Mol. Biol.*, **18**, 68 (1966).

[9] Leder, P., and H. Bursztyn, *Biochem. Biophys. Res. Commun.*, **25**, 233 (1966).

[10] Watson, J. D., *Bull. Soc. Chim. Biol.*, **46**, 1399 (1964).

[11] Mangiarotti, G., and D. Schlessinger, *J. Mol. Biol.*, **20**, 123 (1966).

[12] At high concentration of GTP, the amount of F-met-puro synthesized is greater than the amount of F-met-tRNA bound. This is presumably due to reuse of ribosomes in the synthesis reaction.

[13] Nishizuka, Y., and F. Lipmann, these PROCEEDINGS, **55**, 212 (1966).

[14] Ghosh, H. P., D. Söll, and H. G. Khorana, *J. Mol. Biol.*, **25**, 275 (1967).

[15] Nakamoto, T., and D. Kolakofsky, these PROCEEDINGS, **55**, 606 (1966).

[16] Kolakofsky, D., and T. Nakamoto, these PROCEEDINGS, **56**, 1786 (1966).

[17] Economou, A. E., and T. Nakamoto, these PROCEEDINGS, in press.

[18] Brown, J., J. W. B. Hershey, and R. E. Thach, unpublished experiments.

[19] Nishizuka, Y., and F. Lipmann, *Arch. Biochem. Biophys.*, **116**, 344 (1966).

[20] Nomura, M., and C. V. Lowry, these PROCEEDINGS, **58**, 946 (1967).