THE EFFECT OF DEACYLATED tRNA ON ENZYMATIC BINDING OF N-FORMYL-METHIONYL-tRNA TO RIBOSOMES

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Deacylated tRNA[2] markedly inhibits both AUG-directed binding of f-Met-tRNA to ribosomes (1,2) and the subsequent synthesis of f-Met-puromycin (2) at 10 mM Mg^{++}, in a system to which no soluble factors are added. Since this suggests that under some circumstances, the rate of initiation of protein synthesis may be regulated by the tRNA/f-Met-tRNA ratio, it is of interest to examine the effect of tRNA in an enzymatic binding system. In the present communication, we report that GTP and initiation factors are required for binding of f-Met-tRNA at 5 mM Mg^{++}, as also found by several other investigators (3-7). The results demonstrate that in the presence of GTP and initiation factors, tRNA does not inhibit AUG-directed binding of f-Met-tRNA to ribosomes. A portion of these findings has been presented (8).

METHODS

Materials— E. coli B deacylated tRNA was obtained from General Biochemicals, GMP-PCP, from Miles Chemical Co., and L-^{14}C-methionine (199 μc/μmole), from New England Nuclear. NH_4Cl-washed ribosomes from E. coli B or W3100 were prepared according to Hershey and Thach (9) and were stored in liquid nitrogen. Unfractionated initiation factors F_1 and F_2, obtained from the first NH_4Cl ribosomal wash fluid, were precipitated with 0-70% $(NH_4)_2SO_4$ and then dialyzed

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[2] Abbreviations: tRNA, transfer RNA; f-Met-, Met-, Phe-, N-formyl-methionyl-, methionyl-, phenylalanyl-; $tRNA_f^{Met}$, formylatable species of methionine-accepting tRNA; GMP-PCP, 5'-guanylyl-methylene diphosphonate; AUG, trinucleotide diphosphate ApUpG.

overnight against 0.01 M Tris-HCl, pH 7.6, and 0.01 M β-mercaptoethanol. Unfractionated f-^{14}C-Met-tRNA was prepared as described previously (2). Purified initiation factors F_1 and F_2 and 7 x NH_4Cl-washed ribosomes (each free of transfer factors T and G (10)) from E. coli MRE-600 were the generous gift of Drs. Richard Erbe and Philip Leder (11).

Assay— Each 55-μl. reaction contained 0.05 M Tris-acetate, pH 7.2; 0.05 M NH_4Cl; 5 mM magnesium acetate; 0.15 A_{260} units of AUG (where specified); 1.0 A_{260} units of ribosomes; unfractionated initiation factors (45 μg of protein); 50 mμmoles of GTP or GMP-PCP (as specified); 0.23 A_{260} units of f-^{14}C-Met-tRNA (15 μμmoles); and where indicated, 0.46 A_{260} units of tRNA. Reactions were initiated by the addition of either f-^{14}C-Met-tRNA or a mixture containing both tRNA and f-^{14}C-Met-tRNA. Incubation was for 10 minutes at 24°C. Binding of f-^{14}C-Met-tRNA to ribosomes was assayed by the procedure of Nirenberg and Leder (12). Radioactivity was determined in a Packard scintillation spectrometer (75% counting efficiency).

RESULTS

At 5 mM Mg^{++}, AUG-directed binding of f-^{14}C-Met-tRNA to ribosomes requires initiation factors and GTP (Table 1), and it may be seen that omission of either one or both of these components from complete reactions greatly reduced the level of f-^{14}C-Met-tRNA binding. Addition of a two-fold excess of tRNA to reactions containing initiation factors and GTP (Table 2) had virtually no effect on either binding of f-^{14}C-Met-tRNA to ribosomes[3] or formation of f-^{14}C-Met-puromycin. Similar results were obtained with a tRNA/f-^{14}C-Met-tRNA ratio of eight.

The data of Fig. 1A further illustrate that the rate of f-^{14}C-Met-tRNA binding is unaffected by tRNA in the presence of initiation factors and GTP. In addition, other experiments (not shown) revealed that if tRNA were incubated with ribosomes and AUG for 10 minutes prior to the addition of factors, GTP, and

[3] Both tRNA and Met-tRNA have no effect on f-Met-tRNA binding in the presence of factors and GTP (14).

TABLE 1

Requirements for Binding of f-^{14}C-Met-tRNA to Ribosomes at 5 mM Mg^{++}

Reaction	f-^{14}C-Met-tRNA Bound μμmoles		
	-AUG	+AUG	△
COMPLETE	0.42	1.56	1.14
minus GTP	0.29	0.46	0.17
minus initiation factors	0.06	0.14	0.08
minus GTP and initiation factors	0.09	0.25	0.14

Incubation conditions and assay procedure were as described under Methods, except that 0.86 A_{260} units of ribosomes were added to each reaction.

TABLE 2

Effect of tRNA on Factor-GTP Dependent Binding of f-^{14}C-Met-tRNA to Ribosomes and Formation of f-^{14}C-Met-Puromycin

tRNA	Trinucleotide	f-^{14}C-Met-tRNA Bound		f-^{14}C-Met-Puromycin Formed	
		μμmoles	△	μμmoles	△
None	-AUG	0.33		0.44	
None	+AUG	1.95	1.62	1.86	1.42
+ 2 x tRNA	-AUG	0.28		0.15	
+ 2 x tRNA	+AUG	1.97	1.69	1.53	1.38

Incubation conditions and assay of f-^{14}C-Met-tRNA binding to ribosomes are described under Methods. A duplicate set of reactions containing 50 mμmoles of puromycin were assayed for f-^{14}C-Met-puromycin according to Leder and Bursztyn (13). The μμmoles of f-^{14}C-Met-puromycin formed correspond to the volume of the entire ethyl acetate layer (1.5 ml.).

f-^{14}C-Met-tRNA, it was still unable to inhibit the rate of binding of f-^{14}C-Met tRNA.

To determine whether GTP and initiation factors are both necessary to

prevent inhibition by tRNA, the purified factors F_1 and F_2, rather than an unfractionated mixture, were used. As may be seen from Table 3, tRNA did not reduce f-^{14}C-Met-tRNA binding to ribosomes in reactions containing F_1, F_2, and GTP; however, when GTP was omitted, a two-fold excess of tRNA reduced the binding of f-^{14}C-Met-tRNA by 53%. The inhibition expected from dilution alone is 67%. In agreement with other studies (15,3), binding of f-^{14}C-Met-tRNA in the presence of GTP and F_1 alone was negligible, whereas in the presence of GTP and F_2 alone, binding was 25% of that observed in the complete system. When tRNA was added to reactions containing GTP and F_2, it did not inhibit f-^{14}C-Met-tRNA binding. In the absence of GTP, however, tRNA reduced the binding of f-^{14}C-Met-tRNA as expected on the basis of dilution.

A further investigation of this requirement for GTP was carried out by incubating reactions with GMP-PCP, the β,γ-methylene analogue of GTP (16). Recently, this analogue has been used to demonstrate that hydrolysis of GTP is involved in peptide bond formation, but apparently not in binding; however,

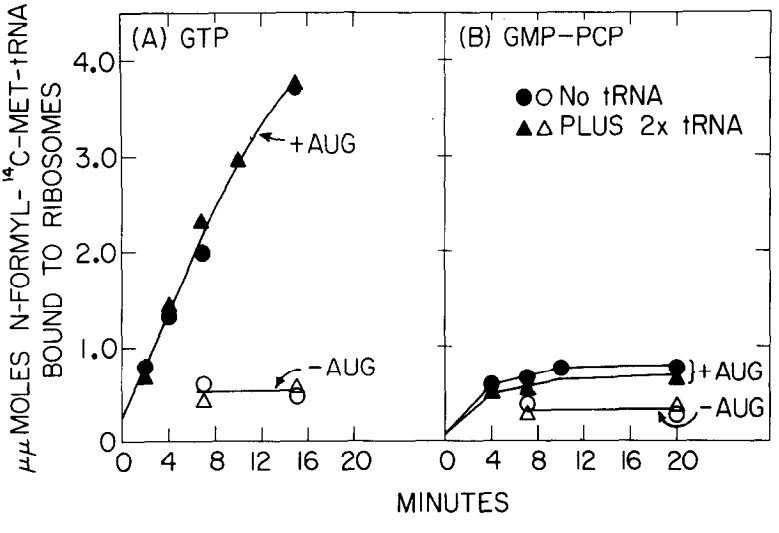

Fig. 1. Effect of tRNA on the rate of f-^{14}C-Met-tRNA binding in the presence of GTP (A) or GMP-PCP (B). Reactions contained the components given under Methods. At the indicated times, portions were withdrawn and binding of f-^{14}C-Met-tRNA to ribosomes assayed as described under Methods. All data were normalized to reaction volumes of 55-μl. Closed symbols, plus AUG; open symbols, minus AUG. —●—●—, —○—○—, minus tRNA; —▲—▲—, —△—△—, plus 2 x tRNA.

TABLE 3

Effect of Purified Factors and GTP on Ability of tRNA to Inhibit f-^{14}C-Met-tRNA Binding to Ribosomes

Addition	Trinucleotide	f-^{14}C-Met-tRNA Bound			
		-tRNA		+ 2 x tRNA	
		μμmoles	△	μμmoles	△
F_1 + F_2 + GTP	-AUG	0.24		0.25	
"	+AUG	5.19	4.95	4.87	4.62
F_1 + F_2	-AUG	0.10		0.10	
"	+AUG	1.50	1.40	0.76	0.66
F_1 + GTP	-AUG	0.04		0.04	
"	+AUG	0.13	0.09	0.10	0.06
F_2 + GTP	-AUG	0.09		0.07	
"	+AUG	1.16	1.07	1.04	0.97
F_2	-AUG	0.08		0.07	
"	+AUG	0.51	0.43	0.16	0.09
F_1 + F_2 + GMP-PCP	-AUG	0.51		0.34	
"	+AUG	1.33	0.82	1.07	0.73

Incubation conditions and assay procedure were as described under Methods, except that 0.60 A_{260} units of 7 x NH_4Cl-washed ribosomes and purified initiation factors F_1 (2.4 μg of protein) and F_2 (2.5 μg of protein) were added to each 50-μl. reaction.

its ability to substitute for GTP in binding of f-Met-tRNA has varied (5,6,17, 18,11). In the experiments reported here, GMP-PCP did not effectively replace GTP. Nevertheless, as shown in Fig. 1B, tRNA had little effect on f-^{14} C-Met-tRNA binding in reactions containing initiation factors and GMP-PCP. Essentially the same results were obtained when the purified factors F_1 and F_2 were used (Table 3). The relatively high background binding of f-^{14}C-Met-tRNA in reactions with F_1, F_2, and GMP-PCP (next to last line, Table 3) is not readily explained. However, this observation, as well as the small effect of tRNA on binding, was confirmed in other experiments.

DISCUSSION

The results indicate that in the presence of initiation factors and GTP, tRNA (presumably $tRNA_f^{Met}$) is unable to compete with f-Met-tRNA for ribosomal binding sites. This is in accord with the observation that at 5 mM Mg^{++}, the formylated derivative of $Met\text{-}tRNA_f$ is required for factor-GTP dependent binding to ribosomes (4,5,19-21). Recent evidence suggests that f-Met-tRNA binds, at least initially, to the 30 S ribosomal subunit (22-4,7,25) at sites for aminoacyl-tRNA (26). Thus, to ensure a unique response to initiator codons, formation of the initiation complex may involve a highly specific interaction between $f\text{-}Met\text{-}tRNA_f$, initiation factors, GTP, and possibly ribosomes, either prior to or during the binding reaction.

In addition to the specificity for $f\text{-}Met\text{-}tRNA_f$, the present study emphasizes the importance of GTP participation in the formation of the initiation complex. Thus, tRNA reduces binding of f-Met-tRNA in reactions without GTP, but has little effect on f-Met-tRNA binding in the presence of GTP (or its β,γ-methylene analogue). These results are similar to the previous finding that with addition of GTP and a soluble enzyme fraction, tRNA inhibition of Phe-tRNA binding is considerably reduced (27,28). Although the present data suggest that in the presence of GTP and initiation factors, $tRNA_f^{Met}$ cannot enter ribosomal sites for f-Met-tRNA, the possibility that GTP facilitates a rapid enzymatic release of bound tRNA cannot be entirely excluded.

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