INHIBITION OF TRANSLATION INITIATION COMPLEX FORMATION BY MS1*

MITSUAKI YOSHIDA, ANDREW TRAVERS and BRIAN F.C. CLARK
M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England

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1. Introduction

When stringent cells of *Escherichi coli* are starved for an amino acid, a guanine nucleotide derivative, MS1, identified as ppGpp [1], rapidly accumulates to relative high concentrations [2, 3]. The level of this derivative usually exceeds that of guanosine triphosphate [3, 4], GTP (pppG), which in turn is in excess over guanosine diphosphate, GDP (ppG) [5]. In general, the level of MS1 can be directly correlated with the rate of cell growth [6]. We show here that MS1 can inhibit a step in protein synthesis; specifically the formation of the initiation complex containing formylmethionyl-$tRNA_f$, 70 S ribosomes and messenger RNA.

2. Methods and results

At 5 mM magnesium ion concentration the formation of the 70 S ribosomal initiation complex requires the presence of initiation factors and GTP [7]. The effect of MS1 on the triplet stimulated binding of fMet-$tRNA_f$ to 70 S ribosomes was studied at 5 mM magnesium ion concentration. The binding of initiator tRNA at the concentration of triplet used is almost independent of the presence of initiation factor IF3 [8, 9]. The results of this experiment using limiting amounts of initiation factors IF1 and IF2 are seen in table 1, expt. 1. The amount of ribosomal complex is measured by the radioactively labelled initiator tRNA bound to nitrocellulose filter (Millipore) adsorbed ribosomes [10]. A concentration of 0.4 mM MS1 inhibits the formation of initiation complex to about 14% of its value in the complete system. This inhibition can be partly reversed by increasing the GTP concentration. Even if the GTP concentration is raised to 0.6 mM where the MS1 concentration is 0.4 mM there is still a substantial (52.8%) inhibition of tRNA binding.

That the inhibition of initiator tRNA binding is dependent upon the role of the initiation factors is seen from the results shown in experiment 2 of table 1. When the experiment is done using 10 mM magnesium ion concentration, the binding of fMet-tRNA, which is minimally dependent upon the presence of GTP at this concentration [11] is not inhibited by MS1.

The inhibition of the binding of fMet-tRNA seen in table 1, expt. 1, depends on the concentration of MS1. This dependence is illustrated by the experimental results plotted in the curve of fig. 1. The range of concentration of MS1 used for this experiment covers a range similar to that observed *in vivo* [3, 4]. This range includes the steady state levels seen during normal growth on various carbon sources and the elevated levels observed in stringent cells during amino acid starvation.

When a natural messenger rather than ApUpG is used to form the initiation complex, an additional initiation factor, IF3, is needed [8, 12]. Table 2 shows the results of adding MS1 to the initiation complex formed with the bacteriophage R17 RNA. There is a clear inhibition of complex formation either with crude factors supplying IF3 (expt. 1 of table 2) or with purified IF3 added to purified IF1 and IF2, [9] (expt. 2 of table 2). In each case, the dependence of complex formation upon presence of IF3 is also shown. In these experiments

* Abbreviation used, MS1 = magic spot 1.

Table 1
Effect of MS1 on ApUpG stimulated binding of fMet-tRNA$_f$ to ribosomes.

	Bound tRNA (pmoles)	Factor stimulation in complete system (%)
Expt. 1: At 5 mM Mg		
Complete,	6.09	100
–IF, –IF2	1.07	0
+MS1 (0.4 mM)	1.79	14.3
+MS1 (0.4 mM) + + GTP (0.4 mM)	3.72	52.8
Expt. 2: At 10 mM Mg		% of ApUpG stimulation
Complete		
–IF1, –IF2, –GTP)	4.65	100
–ApUpG	0.49	0
+MS1 (0.4 mM)	4.55	97.6

Triplet binding assays were performed at 25° with 15 min incubation times as previously described [7]. In expt. 1 the complete system contained 0.1 M Tris-HCl pH 7.4, 0.05 M NH_4Cl, 0.005 M $MgCl_2$, 1.5 μg pure IF1, 2.4 μg pure IF2, 40 pmoles [^{35}S] fMet-tRNA$_f$, 0.05 A_{260} units of ApUpG, 2.2 A_{260} units of 70 S ribosomes (washed with 2 M NH_4Cl) and 0.2 mM GTP. The concentrations of IF1 and IF2 used were limiting. Additions or omissions to the complete system are indicated by + and – signs. In expt. 2, the complete system contained 0.01 M $MgCl_2$ but no GTP, IF1 or IF2. MS1 was purified by thin-layer chromatography as described by Cashel [3]. This nucleotide was estimated to be >95% pure.

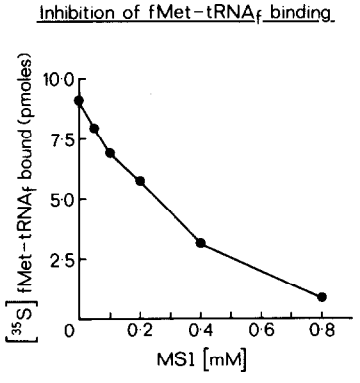

Fig. 1. The amount of inhibition of ribosomal bound fMet-tRNA$_f$ directed by ApUpG was measured at various concentrations of MS1. The binding assay used conditions as described in expt. 1 of table 1. The binding was carried out under limiting IF1 and IF2 and in the presence of 0.2 mM GTP.

Table 2
Effect of MS1 on R17 RNA directed fMet-tRNA$_f$ binding.

	Bound tRNA (pmoles)	Stimulation by factors in complete system (%)
Expt. 1:		
Complete (crude factors)	13.01	100
– crude factors	0.30	0
+ MS1 (1.0 mM)	1.02	5.7
Expt. 2:		
Complete	6.09	100
– IF3	0.45	0
+ MS1 (0.4 mM)	1.88	25.3

The fMet-tRNA$_f$ bound to ribosomes was assayed as for expt. 1 of table 1 except that R17 RNA (1.0 A_{260} unit) replaced ApUpG in the complete system and the incubation temperature was 37° instead of 25°. In expt. 1, 30 μg of a crude factor preparation prepared from the ribosomal wash proteins [19] was used as a source of IF3 required for R17 RNA stimulated binding. In expt. 2 the complete system contained 0.3 μg of pure IF3.

inhibitions were obtained by different concentrations of MS1; 1.0 mM for expt. 1 and 0.4 mM for expt. 2.

An important feature of the inhibition of initiation complex formation is the lack of dissociation of the initiation complex once it is formed. If the initiation complex is pre-formed before the addition of MS1 then there is no reduction in the amount of complex formation (as shown in table 3). Table 3 also shows that a concentration of GDP similar to that of MS1 results in a similar amount of inhibition of initiation complex formation. Addition of GDP after the complex has been preformed also fails to destroy the complex. Table 3 shows that the bound tRNA stable against removal by MS1 is not formed before ApUpG dependent ribosomal binding when ApUpG is added with MS1 after preincubation without ApUpG.

The results presented here could be explained by the generation of GDP from MS1 during the incubation. However in a simulated reaction containing all necessary components ^{32}P labelled MS1 was stable.

Table 3
Effect of MS1 and GDP on triplet directed preformed initiation complex.

Components for 1st incubation	2nd incubation	Bound tRNA (pmoles)	% of complete
Complete	None	4.70	100
– IF1, – IF2	None	0.86	0
– IF1, – IF2, + MS1 (0.3 mM)	None	0.62	–6.2
+ MS1 (0.3 mM)	None	3.07	64.0
Complete	+ MS1 (0.3 mM)	4.17	99.1
– ApUpG	+ ApUpG, + MS1 (0.3 mM)	2.31	44.3
+ GDP (0.3 mM)	None	3.25	69.0
Complete	+ GDP (0.3 mM)	5.01	108

The binding of initiator tRNA to ribosomes was measured as for expt. 1 of table 1 with 0.005 M Mg^{2+} conditions. Both incubation phases indicated were for 10 min at 25°. The additions for the second incubation were as indicated with equivalent water volumes added to the tubes where no further additions were required.

Since there was some inhibition of factor independent binding (results in line 3 of table 3) this figure was used to subtract from the complete system inhibited by MS1 for calculating the percentage of bound tRNA in the complete system for the last column.

3. Discussion

We have shown that MS1 (ppGpp) will inhibit *in vitro* a step in the initiation of protein synthesis. In at least one experiment, that measuring the AUG directed binding of fMet-tRNA to ribosomes, GDP was as effective an inhibitor as MS1. However in the cell MS1 accumulates to levels comparable to or exceeding those of GTP [3], which in turn exceed those of GDP by an order of magnitude [5]. Thus *in vivo* MS1 is more likely than GDP to affect reactions involving a requirement for GTP in protein biosynthesis. GTP has been shown to be concerned in several specific steps of protein synthesis *in vitro* namely initiation, elongation and termination (the last at least for eukaryotic cells). Should MS1 be a general regulator of protein biosynthesis it might interfere with the initiation step, the first event which used GTP. Here we have shown that MS1 does indeed inhibit the binding of initiator tRNA to ribosomes at the initiation step prior to the first peptide bond formation. During the formation of the initiation complex GTP is thought to interact with initiation factor IF2 [13, 14], before the recognition of initiator tRNA for the completion of the ternary complex including $fMet\text{-}tRNA_f$, GTP and IF2 [15, 16]. This ternary complex decodes messenger attached to ribosomes for the initiation step. Possibly MS1 will inhibit these interactions involving GTP and so prevent the recognition of the initiator tRNA for its incorporation into the ribosomal initiation complex. Such an inhibition is probably primarily a kinetic effect.

Blumenthal et al. [17] have shown that the elongation factor Tu will bind MS1 very tightly. Thus the possibility exists that MS1 might also inhibit the elongation step in protein biosynthesis *in vitro*.

Further the ψ activity manifested by the complex of Tu and Ts in the transcription reaction is inhibited by MS1 at concentrations comparable to those used here [18]. If MS1 were to act *in vivo* as it does *in vitro* its role would possibly be that of a general regulator of both RNA and protein synthesis. Such regulation would be dependent in part on the ratio of MS1 to GTP.

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