ENTRY SITE OF FORMYLMETHIONYL-tRNA

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Received 30 November 1971

1. Introduction

Binding of fMet-tRNA to a ribosome–bacteriophage RNA complex, which is mediated by the initiation factors F1, F2 and F3, has been shown to occur either with GTP or with GMP-PCP, β-γ-methylene analogue of GTP [1–7]. The fMet-tRNA in the 70 S initiation complex formed with GTP can react with puromycin, whereas fMet-tRNA in the complex with GMP-PCP cannot [1]. Because the GTP-analogue is non-hydrolysable, the hypothesis has been put forward that a translocation step, which needs GTP-hydrolysis, occurred during complex formation [5]. These results have led to the conclusion that there is one single entry site on the ribosome, the A-site, which functions also for fMet-tRNA [7]. GTP hydrolysis and concomitant translocation positions the fMet-tRNA into the P-site, where it becomes reactive towards puromycin. These observations were also in line with further studies, which dealt with binding of a second aminoacyl-tRNA to initiation complexes, in which either bacteriophage RNA or an AUG_2U_n oligonucleotide was used as mRNA. With bacteriophage MS2-RNA it was shown that alanyl-tRNA could not enter the initiation complex in the presence of GMP-PCP [6], whereas valyl-tRNA binding was completely inhibited with AUG_2U_n [4, 5].

The data we wish to present in this paper, however, can best be explained by assuming that fMet-tRNA enters directly into the P-site, irrespective whether GTP or GMP-PCP is used for complex formation [8, 9]. Our experiments indicate that the puromycin reaction with the initiation complex formed with GMP-PCP fails because of the blocking of the A-site by F2 and GMP-PCP rather than by wrong positioning of the fMet-tRNA itself.

2. Materials and methods

Ribosomes were isolated from *E. coli,* Q13 strain, by alumina grinding as described earlier [6] and washed 2 times with 1 M NH_4Cl. Initiation factor F2 and initiation factor complex F1/F3 were prepared according to Hershey et al. [15]. The fMet-tRNA binding assay was performed in 50 mM K-acetate, 50 mM Tris-HCl, pH 7.2, 7 mM Mg-acetate, 50 mM NH_4Cl and 1.2 mM β-mercaptoethanol (buffer A); the binding was determined by the Millipore filter technique. Formylmethionyl-puromycin was extracted with ethylacetate at pH 5.5 according to Leder and Bursztyn [16]. Post-column initiation complexes were eluted from a 1.0 × 25 cm Sephadex G-200 column with buffer A. The initiation complex containing fractions were pooled and samples of 0.1 ml were used for post-column experiments.

3. Results and discussion

We believe that meaningful studies about the properties of the initiation complex can best be performed on the isolated complex. The advantage of this approach being the absence of all factors which were necessary for its formation. In this way we can exclude the interference of these factors with subsequent relevant reactions, e.g. binding of the second aminoacyl-tRNA or the puromycin reaction. Thus

Table 1
Puromycin sensitivity of fMet-tRNA in initiation complexes.

Pre-column		
Preincubation	Binding (pmoles)	Puromycin sensitive (%)
+ GTP	2.72	86.0
+ GMP-PCP	2.07	18.3
Post-column		
Preincubation	Binding (pmoles)	Puromycin sensitive (%)
+ GTP	1.80	80.0
+ GMP-PCP	2.55	88.3

Pre-column: 0.1 ml incubation mixtures in buffer A contained 120 μg washed ribosomes from *E. coli* Q13, 10 μg of MS2-RNA, 0.2 mM GTP or GMP-PCP, 20 pmoles of f-^{3}H-Met–tRNA (specific activity 7.2 Ci/mM), 1.0 μg of F2 and 0.5 μg of F1/F3-complex and were incubated for 15 min at 37°. To another set of samples 50 μg of puromycin was added subsequently and incubated for another 10 min. Blank values are subtracted.
Post-column: 0.5 ml incubation mixtures containing 840 μg of washed ribosomes, 70 μg of MS2-RNA, 140 pmoles of f-^{3}H-Met-tRNA, 7 μg of F2 and 3.5 μg of F1/F3 were incubated and passed over Sephadex G-200 as described in Materials and methods. 0.1 ml samples of the pooled fractions were directly filtered over Millipore or incubated for another 30 min in the presence of 50 μg of puromycin. Binding of fMet-tRNA and fMet-puro formation were determined as described. The presented figures were calculated per 0.1 ml of the pre-column incubation mixtures.

Table 2
Effect of Sephadex chromatography on A-site bound Phe-tRNA.

Pre-column		
Additions	Binding (pmoles)	Puromycin sensitive (%)
–	1.05	5.0
Post-column		
Additions	Binding (pmoles)	Puromycin sensitive (%)
a. –	1.05	7.9
b. G	1.05	5.7
c. GTP	1.05	25.1
d. GTP + G	1.05	73.2

Pre-column: 0.1 ml incubation mixtures in buffer A, containing 120 μg of ribosomes, 10 μg of poly U, 20 pmoles of ^{14}C-Phe-tRNA (specific activity 514 mCi/mM), 10 μl of 105,000 *g* supernatant, 0.02 μmoles of GMP-PCP and 50 μg of puromycin were incubated at 37°. After 30 min of incubation a constant binding was reached, indicating that all Phe-tRNA bound to the ribosome was puromycin resistent.
Post-column: 0.5 ml incubation mixtures were incubated and passed over Sephadex G-200 as described in Materials and methods. 0.1 ml samples of the pooled fractions were directly filtered over Millipore or incubated for another 30 min in the presence of 50 μg of puromycin, (a); 50 μg of puromycin and 10 μl of 105,000 *g* supernatant, (b); 50 μg of puromycin and 0.02 μmoles of GTP, (c); or 50 μg of puromycin, 0.02 μmoles of GTP and 10 μl of 105,000 *g* supernatant, (d); whereafter the remaining ^{14}C-Phe-tRNA was counted on Millipore. Post-column binding is calculated per 0.1 ml pre-column incubation mixture.

the initiation complex was isolated by gel filtration on Sephadex G-200 columns. These isolated initiation complexes were tested on their ability to perform the formylmethionyl-puromycin reaction. The results of this assay are summarized in table 1. fMet-tRNA bound in the presence of GMP-PCP becomes puromycin sensitive after Sephadex passage, whereas it was non-reactive before. This lead us to assume that GTP hydrolysis was not necessarily needed for positioning the fMet-tRNA in a reactive site. A control experiment was set up to check that column passage by itself cannot convert a pretranslocational complex into a posttranslocational complex. This was achieved by binding Phe-tRNA to a poly U-ribosome complex in the presence of GMP-PCP. Non-enzymic binding in the P-site was removed by puromycin, whereafter the complex was passed through the column. As is shown in table 2 Phe-tRNA becomes puromycin sensitive only after addition of GTP and G factor. The loss of counts in the presence of GTP might be due to residual G activity on the ribosome.

These results lead us to reconsider the initial concepts of fMet-tRNA binding [1–7]. We postulate therefore that non-reactivity of fMet-tRNA apparently is not due to a wrong positioning but more likely to the presence of initiation factors or other components in initiation complexes which do impair normal ribosomal function. The column procedure presumably

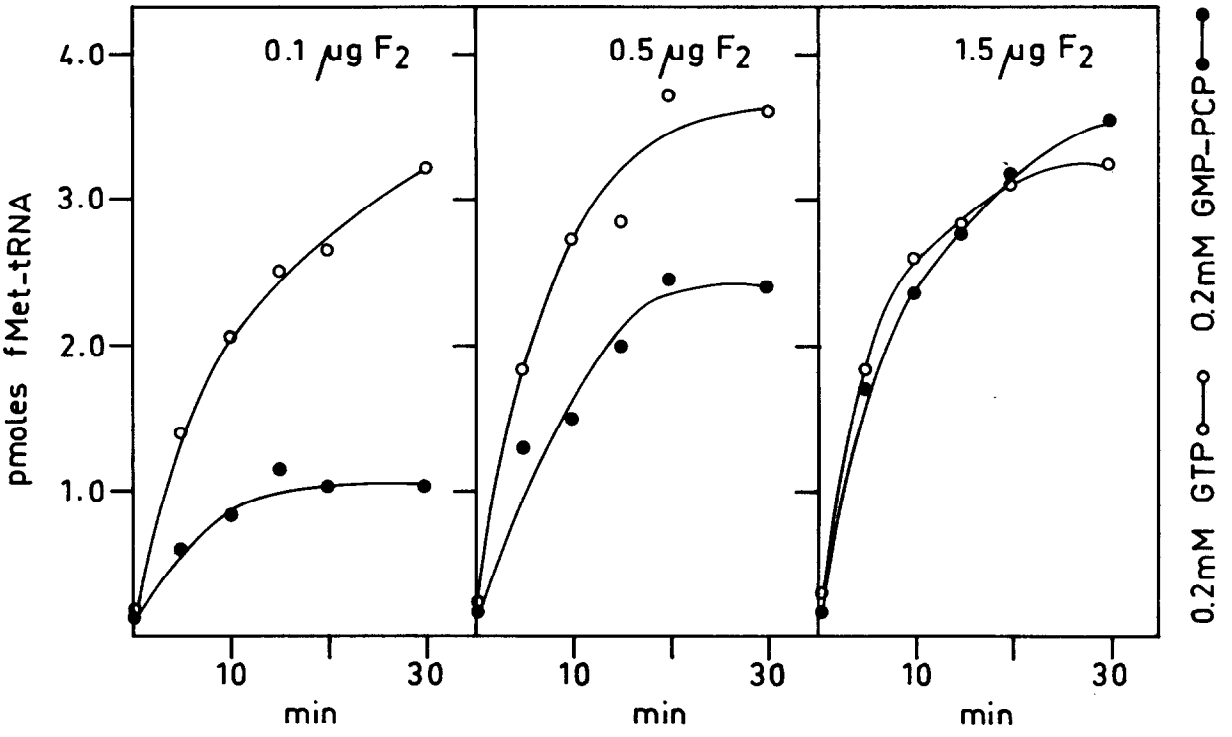

Fig. 1. Kinetics of fMet-tRNA binding with varying amounts of F2. Incubation mixtures and ionic conditions identical to those in table 1, except that the amount of F2 was varied, 0.1; 0.5 and 1.5 µg of F2. (o—o—o): 0.2 mM GTP; (•—•—•): 0.2 mM GMP-PCP.

Table 3
Effect of F2, GTP and GMP-PCP on puromycin sensitivity of fMet-tRNA.

Post-column		
Postincubation Additions	Binding (pmoles)	Puromycin sensitive (%)
none	2.55	90.0
+ F2	2.50	92.1
+ GTP	2.50	90.5
+ GMP-PCP	2.50	89.7
+ F2 + GTP	2.55	89.9
+ F2 + GMP-PCP	2.55	39.1
+ F1 + F2 + GTP	2.55	91.2
+ F1 + F2 + GMP-PCP	2.55	38.7

Post-column complexes were prepared as described in the legend to table 1. Samples of 0.1 ml were submitted to a repeated incubation with 1.0 µg of F2, 0.5 µg of F1, 0.02 µmoles of GTP or GMP-PCP separately or combined as shown, all in the presence of 50 µg of puromycin. The puromycin sensitivity was determined both on Millipore and by extraction with ethylacetate.

removes initiation factors from the complex, thereby causing the reactivation of the puromycin reaction. For this reason we have incubated post-column complexes again with either GTP or GMP-PCP and/or F2. One observes that only F2 and GMP-PCP in a combined action can restore the puromycin insensitivity of these complexes (table 3). F1 does not play any role in this reaction. The results so far presented can be contained in the following scheme. During the formation of the initiation complex with GTP the factors are released. These initiation factors are recycled in a normal incubation mixture. GMP-PCP, however, is thought to prevent or retard this recycling of factors. Evidence for such an assumption could be obtained from experiments in which F2 was added in limiting amounts (see fig. 1). The kinetics of the experiments in fig. 1 show that low amounts of F2 can maintain a reasonable efficient binding of fMet-tRNA, however, only in the presence of GTP. With GMP-PCP both the initial rate and the saturation

Table 4
Effect of F2, GTP and GMP-PCP on ^{14}C-Ala-tRNA binding.

Precolumn incubation	Postcolumn incubation	Binding ^{14}C-Ala-tRNA (pmoles)	
		− F2	+ F2
+ GTP	+ GTP	2.12	2.60
+ GTP	+ GMP-PCP	1.35	0.76
+ GMP-PCP	+ GTP	3.19	3.12
+ GMP-PCP	+ GMP-PCP	1.91	0.50

Postcolumn complexes were prepared with f-^{3}H-Met-tRNA as described before in the presence of either GTP or GMP-PCP. Samples of 0.1 ml were supplied with either 0.02 μmoles of GTP/GMP-PCP and/or 2.5 μg F2, preincubated for 5 min, whereafter 20 pmoles of ^{14}C-Ala-tRNA were added and the Mg-concentration raised to 10 mM. Incubation was continued for another 15 min. Binding of ^{14}C-Ala-tRNA was determined on Millipore filter. Control experiments without column passage: with GTP, 2.15 pmoles ^{14}C-Ala-tRNA bound; with GMP-PCP 0.39 pmoles ^{14}C-Ala-tRNA bound.

Table 5
Effect of tetracycline on Phe-tRNA and f-Met-tRNA binding.

Assay system	Tetracycline	Inhibition of binding (%)	
		+ GTP	+ GMP-PCP
+ poly U	0	0	0
+ poly U	1.0×10^{-4} M	42.5	61.7
+ poly U	2.0×10^{-4} M	61.0	60.3
+ ApUpG	0	0	0
+ ApUpG	1.0×10^{-4} M	0	0
+ ApUpG	2.0×10^{-4} M	0	0

Poly U directed system: see legend to table 2, without tetracycline the amount of ^{14}C-Phe-tRNA binding was 20.6 and 6.5 pmoles with GTP and GMP-PCP, respectively.
AUG-directed system: see legend to table 1, without tetracycline the amount of f-^{3}H-Met-tRNA binding was 1.75 and 2.25 pmoles with GTP and GMP-PCP, respectively.

level of fMet-tRNA binding are at least three times lower. On the other hand excess of F2 gives essentially the same binding level. A likely explanation for these results is that F2 is not recycled with GMP-PCP, acts stoichiometrically, and that GTP hydrolysis might be the driving force for recycling.

So far our hypothesis is based mainly on puromycin reactions. If one assumes that inhibition of the fMet-puromycin reaction is generated by blocking or altering the A-site characteristics by the presence of F2 on the ribosome, one can predict that also the binding of alanyl-tRNA, which is the second aminoacyl-tRNA to be bound in a MS2-RNA–ribosome complex, must be impaired by F2 and GMP-PCP. The data on experiments of this kind are presented in table 4 and are consistent with this hypothesis.

It was already known that in normal incubation mixtures GMP-PCP could inhibit Ala-tRNA binding [6]. However, after passage through Sephadex G-200 both complexes, preincubated with either GTP or GMP-PCP, are able to bind Ala-tRNA. Again addition of GMP-PCP and F2 causes a strong inhibition of this binding reaction. These findings readily suggest that also in these experiments attachment of F2 and probably of GMP-PCP to the initiation complex restricts the ability of the ribosome to perform its normal function, i.e. the presence of F2 in the initiation complex blocks or alters the A-site. From these data we have obtained evidence that the A-site is not involved in the binding of fMet-tRNA. This is supported by experiments in which tetracycline is used [7, 9]. Under conditions where Phe-tRNA binding is significantly inhibited by tetracycline we could not detect any inhibition of fMet-tRNA binding (table 5). Since tetracycline only interferes with binding in the A-site, we like to conclude that fMet-tRNA directly enters the P-site, as was originally proposed by Bretscher, Clark and Marcker [8, 9]. Evidence obtained by other experimental procedures does support this final conclusion [10–13]. The suggested altered P-site, the so-called P-aligned site [14] and the pre-P-site [11] might well correspond to initiation complexes from which the initiation factor has not yet been removed.

Acknowledgements

We thank Professor L. Bosch and Dr. J. van Duin for helpful discussions. This work was sponsored by the Netherlands Foundation for Chemical Research

(S.O.N.), with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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