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SPECIFIC INTERACTION OF INITIATION FACTOR IF2 OF E.COLI WITH FORMYLMETHIONYL-$tRNA_f^{Met}$

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SUMMARY

The interaction of E.coli initiation factor IF2 with formylmethionyl-$tRNA_f^{Met}$ has been studied by measuring the inhibition by IF2 of the spontaneous deaminoacylation of the charged tRNA. We find that IF2 protects fMet-$tRNA_f^{Met}$ against spontaneous deacylation. The formylation is an absolute requirement for this protection and no effect of GTP was found. The association constant for IF2 binding to fMet-$tRNA_f^{Met}$ at 37°C and physiological ionic conditions was estimated at about 10^6 M^{-1}.

The procaryotic initiation factor IF2 plays an important role in the formation of the 30S initiation complex fMet-$tRNA_f^{Met}$:mRNA:30S subunit (1). Many workers regard IF2 as an aminoacyl carrier protein which ensures the correct binding of the first tRNA to the ribosome in a similar manner to the action of the elongation factor EF-Tu on the binding of aa-tRNA to the ribosome during the elongation process. The EF-Tu forms a stable ternary complex with aminoacyl-tRNA and GTP (2). Although GTP has been shown to bind to IF2 and also stabilizes the binding of the factor to the ribosomal 30S subunit (3) no ternary complex fMet-tRNA: IF2:GTP has been isolated.

Apparently contradictory results have arisen from a number of different methods used in a study of this problem. We have previously proposed the existence of such a ternary complex (4). When tritiated GTP was incubated in the presence of IF2 and subsequently applied to a Sephadex G-50 column, radioactivity was eluted in the void volume of the column. The appearance of the GTP in these fractions was completely dependent on the presence of IF2. Moreover, addition of fMet-tRNA produced a five-fold increase in GTP eluted in the high-molecular fractions. These experiments showed that the binding of GTP to IF2 was stimulated by fMet-$tRNA_f^{Met}$ and to a smaller extent by Met-$tRNA_f^{Met}$ but did not establish whether GTP was essential for forming a stable complex between IF2 and the initiator tRNA.

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The same assay was used by Lockwood et al. (5) who showed that the elution of GTP in the tRNA fractions specifically depended on the presence of the initiator tRNA. The unformylated Met-tRNA$_f^{Met}$ bound about half the amount of GTP compared to the formylated species. In addition, Groner and Revel (6) isolated a complex of IF2 and fMet-tRNA$_f^{Met}$ by glycerol gradient centrifugation. The complex could be made only with fMet-tRNA$_f^{Met}$ and not with other aa-tRNAs. The unformylated initiator tRNA was not used in these experiments. Apparently the complex formation did not require GTP and, as in the experiments mentioned hitherto, the salt conditions during incubation were 5-6 mM magnesium chloride and 50-80 mM ammonium chloride.

More recently Majumdar et al. (7) found that radioactive fMet-tRNA$_f^{Met}$ could be retained by nitrocellulose filters when filtered after incubation with IF2 in the presence of buffered 100 mM ammonium chloride. The retention did not require GTP and occurred only in the absence of magnesium. An excess of 40 times IF2 to fMet-tRNA$_f^{Met}$ was needed to retain 60% of the tRNA by the filter. Unformylated Met-tRNA$_f^{Met}$ was not retained by the filters at any IF2 concentration. Shortly afterwards, Sundari et al. (8) also used the filter assay to show that many N-blocked aminoacyl-tRNA's complexed with IF2 on the nitrocellulose in the absence of magnesium and ammonium ions. Formylation of Met-tRNA increased the binding by about 5 times. Van der Hofstad et al. (9) concluded that a binary complex IF2:fMet-tRNA$_f^{Met}$ was formed at conditions optimal for initiation complex formation as such a complex could be isolated on cellulose nitrate filters after fixing with glutaraldehyde. This complex formation did not require GTP and was not inhibited by GDP.

In summary, it was found that IF2 invariably interacted with fMet-tRNA$_f^{Met}$. The extent of interaction with unformylated Met-tRNA$_f^{Met}$ varied from no detectable interaction to almost the same level as for the formylated species. No experiments indicated that the presence of GTP was required for IF2 to interact with initiator tRNA. The ionic conditions for the formation of the binary complex IF2:fMet-tRNA$_f^{Met}$ varied considerably.

A general feature of all the preceding experiments was that they attempted to isolate a macromolecular complex such that the complex may have dissociated during the preparation. Therefore we attempted to study this problem using a method which did not require isolation of the complex.

We used the spontaneous chemical hydrolysis of the amino acid ester bond in aminoacyl-tRNA as a measure of the accessibility of this linkage in a similar way to the studies by Pingoud et al. (10) on the interaction of EF-Tu and aminoacyl-tRNA. It was expected that binding of IF2 at, or near, the C-C-A stem of the tRNA would result in protection against hydrolysis thereby increasing the half life of the ester bond.

The results show that IF2 specifically inhibits the hydrolysis of fMet-tRNA$_f^{Met}$. GTP is not needed for the protection and no effect of IF2 is seen on the deaminoacylation of Met-tRNA$_f^{Met}$ or the non initiator Met-tRNA$_m^{Met}$. The IF2 effect is found at magnesium concentrations of 0-20 mM and monovalent cation concentrations up to 0.7 M.

MATERIALS AND METHODS

Initiation factor IF2 was isolated from E.coli MRE600 and purified by M. J. Dondon as described elsewhere (11). When analysed by SDS-polyacrylamide gel electrophoresis one band corresponding to about 100,000 daltons contained approximately 95% of the total protein applied.

Partially purified tRNA^{Met} (from Microbiological Research Establishment, Porton Down, England) was further purified on BD-cellulose (12). The tRNA$_f^{Met}$ or tRNA$_m^{Met}$ was charged with Methionine (^{3}H-methyl, 0.9 Ci/mmol from Amersham, England) as described (13) with a yield of 1000-1300 pmol Methionine/A$_{260}$ unit of tRNA. Met-tRNA$_f^{Met}$ was formylated chemically by the formyl ester of N-hydroxysuccinimide (14), the degree of formylation was 98%.

The binding of IF2 to fMet-tRNA$_f^{Met}$ was assayed by the effect of the factor on the spontaneous chemical hydrolysis of the amino acid ester bond. fMet-tRNA$_f^{Met}$ (or other tRNA species as indicated in the legends) was incubated at 37°C in the following buffer: Tris-HCl pH 7.5, 50 mM, potassium chloride, 150 mM, 2-mercaptoethanol, 7 mM, magnesium chloride, 6 mM if not other otherwise indicated and IF2 as indicated.

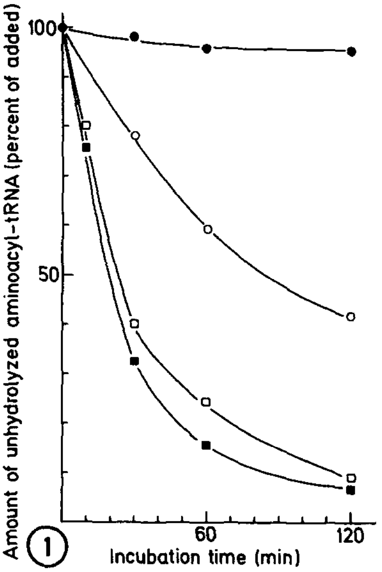
Aliquots of the incubation mixtures were spotted on Whatman 3MM filters, washed twice with 10 ml 5% TCA, rinsed in ethanol, dried and counted in toluene-PPO-POPOP in a Beckman L250 Liquid Scintillation Counter. Reference experiments were incubated for 30 sec. and treated as described.

RESULTS

Aminoacyl-tRNA is a compound whose stability depends strongly on the environmental conditions. At pH around 5 and at low temperature little hydrolysis is seen after months of storage, whereas rapid hydrolysis occurs above pH 8 and at elevated temperature. Thus it is possible to select conditions of pH and temperature where the half life of the ester bond is of the order of minutes, i.e. the usual incubation time in *in vitro* experiments.

At pH 7.5, 37°C, 6 mM magnesium chloride and 150 mM potassium chloride the half life of Met-tRNA$_f^{Met}$ and fMet-tRNA$_f^{Met}$ is 20 and 80 min respectively (open symbols in Fig.1.). An incubation time of 2 hr at these conditions will therefore give a sufficiently high degree of discharge to detect whether IF2 has any effect on the rate of hydrolysis. Fig.2 shows the amount of unhydrolyzed ester bond after 2 hr as a function of IF2 concentration. The initiation factor has no effect on the hydrolysis of unformylated initiator Met-tRNA$_f^{Met}$ or on the non initiator Met-tRNA$_m$ whereas the formylated initiator tRNA is almost completely protected against hydrolysis in the presence of 9 times excess of IF2 to tRNA. The same effect is shown by the closed symbols in the kinetic experiment in Fig.1.

It is well known that IF2 sticks to both glass and plastic surfaces and although carefully siliconized glass tubes were used in our experimentsabout 30% of the



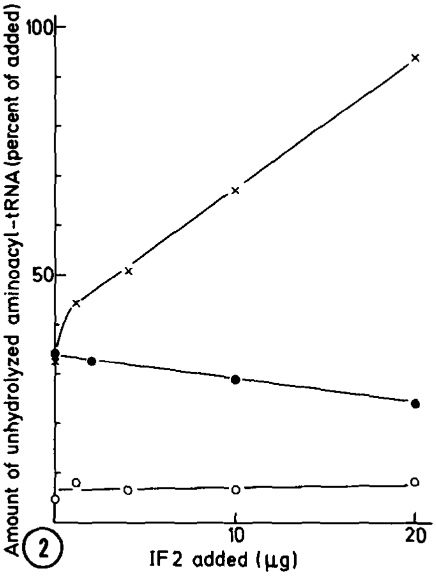


Fig. 1. Kinetics of the non enzymatic hydrolysis of the aminoacyl ester bond of fMet-tRNA_f^{Met} and Met-tRNA_f^{Met} at 37°C.
The incubation conditions were described in Materials and Methods. The total volume was 250 µl and contained fMet-tRNA_f^{Met}, 0.44 µM in the absence of IF2 (o) and in the presence of 3.2 µM IF2 (●) or Met-tRNA_f^{Met}, 0.52 µM in the absence of IF2 (□) and in the presence of 3.2 µM IF2 (■).
Samples of 45 µl were taken after the times indicated and treated as described in Materials and Methods.

Fig. 2. Effect of IF2 on the non enzymatic hydrolysis of the aminoacyl ester bonds of fMet-tRNA_f^{Met}, Met-tRNA_f^{Met} and Met-tRNA_m^{Met}.
Reaction mixtures of 50 µl were incubated for 2 hr at 37°C. The conditions were as in Fig. 1 for fMet-tRNA_f^{Met} (x) and Met-tRNA_f^{Met} (o). The concentration of Met-tRNA_m^{Met} was 0.52 µM (●) and the IF2 concentration is indicated.

added tRNA was lost when IF2 was present during incubation, presumably because of binding to IF2 attached to the tube wall. We think that a similar explanation can be given for the results from earlier nitrocellulose filter assays (7,8). Only at very low ionic strengh will IF2 bind to the nitrocellulose and retain fMet-tRNA_f^{Met}. The fact that no stoichiometric complex could be isolated on the filters also indicates that the observed salt effect was on an IF2 binding to the filters rather than on a binding to the tRNA.

In our experiments 6 mM magnesium chloride and 150 mM potassium chloride are standard conditions, but the protective effect of IF2 on the hydrolysis of fMet-tRNA_f^{Met} is found at magnesium concentrations in the range of 0 to 20 mM (Table 1). The results also show rather interestingly that magnesium catalyses the non enzymatic hydrolysis both in the absence and in the presence of IF2. In some experiments we used up to 0.7 M potassium chloride. Here the presence of a five-fold excess of IF2 to fMet-tRNA_f^{Met} inhibited the hydrolysis after 2

Table 1

Hydrolysis of the aminoacyl ester bond in fMet-tRNA$_f^{Met}$, the effect of IF2 and magnesium concentration

$MgCl_2$ concentration mM	% hydrolyzed after 2 hours - IF2	+ 4 μM IF2
0	38	0
6	55	4
20	66	18

The incubation conditions were described for fMet-tRNA$_f^{Met}$ in Fig. 2.

hr at 37°C from a figure of 90% to 60%. The effect of increased concentration of potassium chloride was the same as found with magnesium chloride (results not shown).

Finally we have studied the effect of GTP on the discharge of fMet-tRNA$_f^{Met}$ at different IF2 concentrations. The results in Table 2 show that GTP has no significant effect in this assay. The presence of GDP or GTP plus a GTP regenerating system (phosphoenolpyruvate and phosphokinase) had no effect on the protection by IF2 (results not shown) indicating that the lack of additional protection by GTP was not caused by an inhibitory effect of GDP.

From Fig. 2 an association constant for the binding of IF2 to fMet-tRNA$_f^{Met}$ can be estimated at about 10^6 M^{-1} at 37°C, pH 7.5, 6 mM magnesium chloride and 150 mM potassium chloride.

Table 2

Effect of GTP on the hydrolysis of the aminoacyl ester bond of fMet-tRNA$_f^{Met}$

IF2 μg	% hydrolyzed after 2 hours - GTP	+ 0.5 mM GTP
0	63	64
1	56	49
10	34	40

The incubation conditions were described in legend to Table 1.

DISCUSSION

The protection by proteins against spontaneous hydrolysis of aminoacyl-tRNA has been used as a method to study the interaction of the elongation factor Tu with aminoacyl-tRNAs but not previously in the study of preinitiation processes. A strong effect of a protein factor on the hydrolysis of the aminoacyl-tRNA ester bond can be expected only if the protein interacts at or near this site of the tRNA molecule or if the binding of the protein results in a significant change in the conformation of the amino acid attachment site.

This was found to be the case of EF-Tu binding to different aminoacyl-tRNAs (10) the protective effect being dependent upon the presence of a free α-amino group and GTP.

When fMet-$tRNA_f^{Met}$ is incubated at conditions where the half life of the aminoacyl-tRNA ester bond is normally 80 min, the presence of a 9-fold molar excess of IF2 to tRNA results in only about 2% hydrolysis after 2 hr. The excess of IF2 needed to get this drastic effect is much smaller than the excess needed to bind fMet-tRNA to nitrocellulose filters (7). There a 15-fold excess resulted in the retention of only 25% of the fMet-tRNA and a 40-fold excess was required to bind 60% of the added tRNA to the filters.

Our results show also that IF2 specifically protects the formylated species of the initiator tRNA and that such a protection is independent of the magnesium concentration in the range of 0-20 mM. Furthermore, using this assay we could not show any significant dependence of the IF2 protective effect on the presence of GTP. It must be stressed that our results do not exclude the posibility that GTP is involved in a preinitiation complex with IF2 and fMet-tRNA, we can only conclude that no effect of GTP can be detected by the method used in these experiments. It is concluded, therefore, that IF2 binds to the initiator fMet-$tRNA_f^{Met}$ under physiological ionic conditions in the absence of GTP and that this binding most probably is at or near the C-C-A terminus.

This is in good agreement with the results of Van der Hofstad et al. (9) who could isolate a binary complex IF2:fMet-$tRNA_f^{Met}$ on nitrocellulose filters after glutaraldehyde fixation. This complex was formed independently of GTP or GDP.

We have earlier reported that unformylated Met-$tRNA_f^{Met}$ can be bound to 30S ribosomal subunits stimulated by initiation factors (14). IF2 alone did not stimulate the binding significantly, the maximal stimulation was found in the presence of all three initiation factors. Together with the results reported here this may indicate that a binding of the initiation factors to the 30S ribosomal subunit activates a ribosomal binding site for the unformylated initiator Met-$tRNA_f^{Met}$.

A binding constant for IF2 to fMet-$tRNA_f^{Met}$ was estimated from our results to be about 10^6 M^{-1}. This shows that the affinity of IF2 to the initiator tRNA is of the same order as the affinity of EF-Tu:GTP to some aminoacyl-tRNAs, which was about 4 x 10^6 M^{-1} for Tyr-$tRNA^{Tyr}$ under similar conditions (10).

Further investigation is needed to define the role of this putative complex between the initiation factor IF2 and the initiator fMet-$tRNA_f^{Met}$ more precisely with respect to the ribosomal initiation complex formation. Experiments are in progress in attempt to discover more details of this macromolecular recognition process.

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