PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES: CHARACTERISTICS OF A Met-tRNA$_f^{Met}$ BINDING FACTOR

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Summary

Three factors (IF_1, IF_2, IF_3) resolved by DEAE-cellulose column chromatography of the crude reticulocyte ribosomal salt wash (I fraction), stimulated poly r(U-G) directed methionine transfer from Met-tRNA$_f^{Met}$ into the terminal positions of the synthesized polypeptides. All three factors were necessary for maximum methionine transfer.

A protein factor that is also present in the I fraction and elutes similarly to IF_1 on DEAE-cellulose chromatography, binds Met-tRNA$_f^{Met}$ in the presence of GTP. The complex formed is quantitatively retained on Millipore filter. This complex formation is specific for Met-tRNA$_f^{Met}$. Other amino acyl tRNAs tested such as Met-tRNA$_m^{Met}$, Phe-tRNA^{Phe} and Val-tRNA^{Val} do not form a similar complex. With Met-tRNA$_f^{Met}$, complex formation does not require Mg^{++} ion or AUG codon and is inhibited by the addition of ribosomes.

Recently, we described a peptide chain initiation assay using preincubated reticulocyte ribosomes, I fraction and poly r(U-G) messenger. In this system, the transfer of methionine from Met-tRNA$_f^{Met}$ in response to poly r(U-G) messenger (which presumably synthesizes Met(Cys-Val)$_n$) was dependent on the addition of I fraction (1). DEAE-cellulose chromatography of the I fraction, separated at least three factors (IF_1, IF_2, IF_3) that stimulated poly r(U-G) directed methionine transfer from Met-tRNA$_f^{Met}$. All three factors were necessary to obtain maximum transfer of methionine (2). The roles of these factors in the over-all process of peptide chain initiation are not known.

In this communication, we describe a factor present in the IF_1 preparation that binds specifically Met-tRNA$_f^{Met}$ in the presence of GTP. The complex

formation is assayed by its retention on Millipore filter. This complex formation does not require Mg^{++} ion, and is inhibited by the addition of ribosomes.

Materials and Methods

Rabbit reticulocyte tRNA preparation used in these experiments were prepared as described previously (3). Partially purified $tRNA_m^{Met}$ was prepared by DEAE-Sephadex A-50 column chromatography of crude rabbit liver tRNA (4). As noted previously (4), such a preparation is contaminated with the $tRNA_f^{Met}$ species (approximately 9 per cent). Crude E. coli tRNA was purchased from General Biochemicals.

Unfractionated reticulocyte tRNA was charged with [^{35}S] methionine using E. coli synthetase (4) which charges only the $tRNA_f^{Met}$ species. [^{14}C] Phe-$tRNA^{Phe}$ (retic.) and [^{14}C] Val-$tRNA^{Val}$ (retic.) were prepared from crude reticulocyte tRNA using reticulocyte synthetase and [^{14}C] amino acids (4). Reticulocyte synthetase was also used to charge purified $tRNA_m^{Met}$ with [^{35}S] methionine. E. coli synthetase was used to charge crude E. coli tRNA with [^{14}C] phenylalanine.

Trinucleoside diphosphate A_pU_pG was purchased commercially from Sigma Chemical. Other materials and methods used in these experiments were the same as described previously (1-4).

Results

Fig. 1 describes a typical DEAE-cellulose chromatographic pattern of crude I fraction. As before (2), at least three factors (IF_1, IF_2, IF_3) stimulated poly r(U-G) directed methionine transfer from Met-$tRNA_f^{Met}$. The column fractions were also assayed using the Millipore filtration method, for their abilities to bind Met-$tRNA_f^{Met}$ in the presence of GTP. As shown in Fig. 1, this activity elutes similarly to IF_1, although significant binding

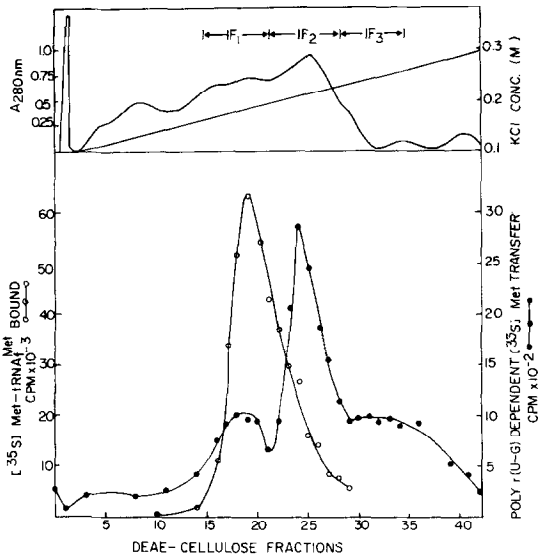

Fig. 1. DEAE-cellulose column chromatography of I fraction and characterization of Met-$tRNA_f^{Met}$ binding factor and IF_1, IF_2 and IF_3. The preparation of I fraction and the DEAE-cellulose column chromatographic fractionation of the initiation factors were as previously described (1-2). The upper curve represents the optical density profile of the column fractions. The lower curves represent (a) the ability of these fractions to stimulate poly r(U-G) directed methionine transfer from [^{35}S] Met-$tRNA_f^{Met}$ into polypeptides •-•-•-•, and (b) the ability to bind [^{35}S] Met-$tRNA_f^{Met}$ in the presence of GTP o-o-o-o-o. A 15 µl aliquot of each fraction and 5.0 pmoles [^{35}S] Met-$tRNA_f^{Met}$ (110,000 cpm) were used in each assay. The assay procedure for poly r(U-G) directed methionine transfer from [^{35}S] Met-$tRNA_f^{Met}$ has been described. [^{35}S] Met-$tRNA_f^{Met}$ binding was assayed using the Millipore filtration technique. Standard incubation mixture contained (in a total volume of 0.075 ml) the following: 20mM Tris-HCl (pH 7.5), 100 mM potassium chloride, 2mM dithiothreitol, 0.2mM GTP, 5 pmoles [^{35}S] Met-$tRNA_f^{Met}$ (110,000 cpm) and 0.015 ml of each column fraction. The incubation was started by adding [^{35}S] Met-$tRNA_f^{Met}$. The reaction mixture was incubated at 37° for 5 minutes, at which time the reaction was terminated by addition of 3 ml cold washing buffer (20mM Tris-HCl (pH 7.5), 100mM potassium chloride, and 2mM dithiothreitol). The solution was then filtered under suction through a Millipore filter (0.45µ). The filter was washed 4 times with 3 ml cold washing buffer. The filter was then dried and counted for radioactivity.

TABLE I

Specificities of the Binding Protein for Amino Acyl tRNAs

Amino Acyl tRNA Used	Total Radioactivity Added (cpm)	Amino Acyl tRNA Bound (cpm)		
		-Factor +GTP	+Factor -GTP	+Factor +GTP
Retic.[^{35}S]Met-tRNA$_f^{Met}$	115,700	310	7,200	68,500
Retic.[^{35}S]Met-tRNA$_f^{Met}$	5,700	40	1,300	4,460
Liver [^{35}S]Met-tRNA$_m^{Met}$	5,400	110	430	630
Retic.[^{14}C]Phe-tRNA^{Phe}	4,000	50	130	90
E. coli [^{14}C]Phe-tRNA^{Phe}	22,000	70	160	180
Retic.[^{14}C]Val-tRNA^{Val}	5,000	60	50	60

Standard binding conditions as described in Fig. 1 were used. Approximately 9 µg of the combined DEAE-cellulose fractions 19-21 were used in each experiment. Specific activities of the radioactively labelled amino acids used in these experiments were: [^{35}S] methionine, 22,000 cpm per pmole; [^{14}C] phenylalanine, 540 cpm per pmole; [^{14}C] valine, 300 cpm per pmole.

activity was also noted in the IF_2 region. The binding activity observed in the IF_2 region may be due to trailing of the IF_1 protein. Further studies will be necessary to decide this possibility.

Table I describes the specificities of the binding protein for amino acyl tRNAs. In these and subsequent experiments, combined peak fractions corresponding to both IF_1 and the Met-tRNA$_f^{Met}$ binding protein (Fractions 19-21) were used. Significant binding was observed only when Met-tRNA$_f^{Met}$ was used. Approximately 60-80 percent of the charged Met-tRNA$_f^{Met}$ species added was bound under the incubation conditions. Some binding of Met-tRNA$_m^{Met}$ observed in these experiments is probably due to the presence of Met-tRNA$_f^{Met}$ species in the Met-tRNA$_m^{Met}$ preparation.

TABLE II

Effects of the Additions of Ribosomes and AUG Triplet Codon on [^{35}S] Met-tRNA$_f^{Met}$ Binding

Experiment	Ribosomes Added (A_{260} Unit)	[^{35}S] Met-tRNA$_f^{Met}$ Bound (cpm) $-Mg^{++}$ -AUG	$-Mg^{++}$ +AUG	$+5mM\ Mg^{++}$ -AUG	$+5mM\ Mg^{++}$ +AUG
A(+Factor)	None	60,500	66,510	25,830	29,050
	0.12	34,710	31,590	21,330	18,440
	0.24	21,100	20,450	19,760	14,200
	0.6	12,880	12,210	11,640	6,940
B(-Factor)	None	110	140	120	150
	0.12	110	820	170	260
	0.24	90	710	160	310
	0.6	100	750	150	550

Standard binding conditions as described in Fig. 1 and Table 1 were used. Experiment A contains in each reaction mixture (total volume, 0.075ml) 9 µg of the combined DEAE-cellulose fractions 19-21. Where indicated 0.16 A_{260} unit of AUG codon and preincubated reticulocyte ribosomes (amount added is shown in parenthesis) were used.

For maximum binding of Met-tRNA$_f^{Met}$ GTP was necessary, although significant binding of Met-tRNA$_f^{Met}$ was also observed in its absence. The stimulatory effect of GTP on the binding reaction was rather specific. Addition of ATP in the absence of GTP produced only slight stimulation of the binding reaction (approximately 50 per cent above background) and UTP and CTP were not stimulatory.

The Met-tRNA$_f^{Met}$ binding reaction apparently does not require Mg^{++} ion and the binding was inhibited by Mg^{++} above 3mM. The binding activity was heat labile and was completely destroyed by heating at 90° for 3 minutes.

We studied the effects of the additions of ribosomes and AUG triplet

TABLE III

Effects of the Additions of Ribosomes on Preformed [^{35}S] Met-tRNA$_f^{Met}$ Complex

Additions	[^{35}S] Met-tRNA$_f^{Met}$ Bound at 2 min	5 min	10 min
		(cpm)	
None	62,500	65,370	67,900
0.24 A_{260} unit ribosomes added at 0 min	10,870	20,500	22,590
2 min	--	47,310	39,390
5 min	--	--	43,600

Reaction conditions were the same as described in Table II. At the indicated times, ribosomes were added.

codon on the binding reaction (Table II). In the absence of any added factor (Expt. B), slight stimulation of Met-tRNA$_f^{Met}$ binding to the ribosomes was observed in the presence of triplet AUG codon. However, this codon directed binding of Met-tRNA$_f^{Met}$ to the ribosomes was very low compared to the binding of Met-tRNA$_f^{Met}$ to the binding factor (Expt. A). Addition of AUG codon alone to the reaction mixture containing the binding factor, consistently produced some stimulation of Met-tRNA$_f^{Met}$ binding (approximately 10 per cent). On the other hand, addition of ribosomes, both in the presence and absence of AUG codon, produced drastic lowering of Met-tRNA$_f^{Met}$ binding. The inhibitory effect of ribosomes on Met-tRNA$_f^{Met}$ binding increased with increasing amounts of added ribosomes.

Table III describes the effects of the additions of ribosomes on the preformed Met-tRNA$_f^{Met}$ complex. As shown in line 1, Table III, the formation of the Met-tRNA$_f^{Met}$ complex with the factor, is over 90 per cent complete within 2 minutes and the complex is stable under the incubation condition for at

least 10 minutes. In these experiments, ribosomes were added at different times during the incubation, and the effect of the added ribosomes on the complex was determined. Addition of ribosomes at 0 min, drastically lowered the rate and extent of the complex formation. Addition of ribosomes after 2 and 5 minutes of preincubation also lowered the extent of Met-$tRNA_f^{Met}$ binding. However, this lowering of Met-$tRNA_f^{Met}$ binding by added ribosomes was less pronounced and could possibly be due to Met-$tRNA_f^{Met}$ deacylase activity present in ribosomes (unpublished observation). These experimental results suggest that ribosomes probably inhibit the initial formation of the Met-$tRNA_f^{Met}$ complex and do not catalyze the conversion of the Met-$tRNA_f^{Met}$ complex into a form not retained on Millipore filter.

Discussion

The DEAE-cellulose chromatographic pattern of the Met-$tRNA_f^{Met}$ binding factor is similar to that of IF_1. As reported previously (2), IF_1 is necessary for poly r(U-G) directed methionine transfer from Met-$tRNA_f^{Met}$. The results presented in this paper, therefore, suggest a possible function of IF_1, namely specific recognition of Met-$tRNA_f^{Met}$ and formation of a Met-$tRNA_f^{Met}$ GTP-IF_1 complex. However, the role of this complex in the overall process of peptide chain initiation is not clear at present. Addition of ribosomes markedly inhibits this complex formation. Further work will be necessary to understand the nature of this inhibition and how the Met-$tRNA_f^{Met}$ complex interacts with ribosomes and cognate codons.

Irvin and Hardesty (5) recently described the presence of a factor in the 0.5M KCl wash of reticulocyte ribosomes which catalyzes the binding of Met-$tRNA_f^{Met}$ to 80S ribosomes in the presence of GTP. This complex formation was also studied by its retention on Millipore filter. Further work will be necessary to compare the Met-$tRNA_f^{Met}$ binding factor described in this paper with the factor described by Irvin and Hardesty (5). Our binding factor does not require ribosomes for activity and is inhibited by their presence.

On the other hand, the Met-$tRNA_f^{Met}$ binding activity described in this paper appears similar to the Met-$tRNA_f^{Met}$ binding factor present in the ribosomal salt wash of L-cells as reported by Levin and Kyner (6). This binding factor forms a ternary complex with Met-$tRNA_f^{Met}$ and GTP and the complex is retained on cellulose acetate membranes. This complex formation is also inhibited by addition of ribosomes.

The Met-$tRNA_f^{Met}$ binding factor described in this paper bears some analogy to the E. coli initiation factor IF_2. Recent reports (7-8) indicate that IF_2 makes a ternary complex with f-Met-$tRNA_f^{Met}$ and GTP. However, unlike the Met-$tRNA_f^{Met}$ complex described in this paper, E. coli f-Met-$tRNA_f^{Met}$-GTP-IF_2 complex is not retained on the Millipore filter. In the presence of the 30S ribosomal subunit, IF_1 and AUG codon, f-Met-$tRNA_f^{Met}$-GTP-IF_2 complex forms the [IF_1, IF_2, 30S, GTP, f-Met-$tRNA_f^{Met}$, AUG] complex which is quantitatively retained on Millipore filters (8).

E. coli initiation factor IF_2 also catalyzes the binding of f-Met-$tRNA_f^{Met}$ to ribosomes in response to AUG codon. The codon directed binding of f-Met- $tRNA_f^{Met}$ to E. coli ribosomes was also studied using Millipore filtration technique. However, in the reticulocyte system, the Met-$tRNA_f^{Met}$ binding factor almost quantitatively binds Met-$tRNA_f^{Met}$ in the absence of ribosomes and the resulting complex is retained on Millipore filters. Addition of ribosomes to the binding reaction inhibits this complex formation. It has therefore, not been possible to ascertain, using the Millipore filtration technique, if the crude I fraction or the purified binding factor described in this paper, stimulates AUG directed binding of Met-$tRNA_f^{Met}$ to reticulocyte ribosomes.

Note Added - The results of recent experiments done in our laboratory suggest that the Met-$tRNA_f^{Met}$-initiation factor-GTP complex formation, as described in this paper, is an essential step in the transfer of methionine from Met-$tRNA_f^{Met}$ into the terminal positions of the polypeptides synthesized in response to poly r(A-U-G) messenger.

A similar Met-$tRNA_f^{Met}$ binding activity in the reticulocyte ribosomal salt wash fraction has also been observed by J. L. Detpman and W. Stanley Biochem. Biophys. Acta. (in press).

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