PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES XIII*: LACK OF mRNA (POLY r(A)) BINDING ACTIVITY IN HIGHLY PURIFIED EIF-1†

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Summary

Met-tRNA$_f^{Met}$ binding factor (EIF-1) has been purified more than 100 fold over crude high salt (0.5 M KCl) ribosomal wash. The purified factor binds 2 nmoles Met-tRNA$_f^{Met}$ per mg protein and shows very little poly r(A) binding activity. Crude ribosomal high salt wash possesses significant amounts of poly r(A) binding activity and also binds to other RNAs. The bulk of this unspecific RNA binding protein is separated from EIF-1 by DEAE-cellulose chromatography.

The first step in peptide chain initiation in eukaryotic cells is the formation of a stable ternary complex between a specific initiation factor, EIF-1, Met-tRNA$_f^{Met}$, and GTP (1-13). This ternary complex formation is specific for Met-tRNA$_f^{Met}$; uncharged tRNA$_f^{Met}$ or other amino acyl tRNAs, including E. coli fMet-tRNA$_f^{Met}$ (12-13), do not form a similar complex with EIF-1.

Recently, Kaempfer (14) and Hellerman and Shafritz (15) have reported that purified EIF-1 (MP preparation of Anderson et. al ref. 9) also binds mRNAs. Hellerman and Shafritz (15) claim that this binding of EIF-1 is most efficient when poly r(A) messenger is used. These authors postulate that EIF-1 has dual functions; EIF-1 binds the initiator tRNA and also eukaroytic mRNAs, presumably through the poly r(A) segment of these messengers. Because of the importance of this observation of Hellerman and Shafritz in understanding the basic mechanism of peptide chain initiation in eukaryotic cells, we have used purified EIF-1 preparation to investigate any possible interaction between EIF-1 and poly r(A) messenger.

MATERIALS AND METHODS

[^{35}S] Methionine (150 to 200 Ci per mmole) was purchased from Amersham-Searle and was diluted with unlabeled methionine to 10,000 to 20,000 cpm per

*Paper XII in this series is Ref. 1.

†Abbreviation: EIF-1, eukaryotic initiation factor 1.

pmole. [^{3}H] Poly r(A) (94 µCi/µmole P) was purchased commercially from Miles Laboratories.

Met-tRNA$_f^{Met}$ binding to EIF-1 was assayed using a Millipore filtration method as previously described (2-4).

[^{3}H] Poly r(A) binding to protein factors was assayed using a Millipore filtration assay method similar to that described by Hellerman and Shafritz (15). The incubation mixtures contained (in a total volume of 0.075 ml) the following: 20 mM Tris-HCl (pH 7.4), 50 mM potassium chloride, 2 mM dithiothreitol, 10 µg bovine serum albumin, 0.06 A_{260} unit (30,000 cpm) [^{3}H] poly r(A) and protein fractions as described in the text. The incubation was at 37° for 5 minutes. The reactions were stopped by addition of 3 ml cold washing buffer containing 20 mM Tris-HCl (pH 7.5), and 50 mM potassium chloride. The solutions were then filtered through Millipore filters and the radioactivity retained on the Millipore filters were counted as previously described (2-4).

Other materials and methods were the same as described previously (2-4).

RESULTS

Purification of EIF-1

The crude 0.5 M KCl ribosomal wash was prepared from anemic rabbit blood cells by the procedure described previously (3-4). The salt wash was dialyzed overnight against Buffer D (5 mM Tris HCl, pH 7.5, 0.1 M KCl, 1 mM dithiothreitol, 50 µM EDTA, and 10% glycerol).

The dialyzed Fraction I (approximately 45 ml, 20 mg/per ml from twelve rabbits) was applied to a DEAE-cellulose (DE-11 Whatmann) column, (2.2 x 10 cm) previously equilibrated with Buffer D. The 0.1 M KCl wash fraction (Fraction IIA) contains the bulk of the poly r(A) binding protein. The column was then washed with 30 ml of Buffer D. The peptide chain initiation factors were then eluted from the column with Buffer D containing 0.3 M KCl (Fraction IIB).

To the 0.3 M KCl eluent (approximately 30 ml) solid ammonium sulfate (enzyme grade) was slowly added with stirring to make the final solution 80% saturated with ammonium sulfate. The ammonium sulfate suspension was stirred for 15 minutes and then centrifuged at 12,000 x g for 15 min. The precipitate was suspended in a minimum volume (10 ml) of Buffer D and the suspension was dialyzed overnight against Buffer D with one change of dialyzing buffer.

The dialyzed 80% ammonium sulfate fraction (approximately 20 ml) (Fraction II) was applied to a DEAE-cellulose (Whatman DE-52) column (1.2 x 12 cm) previously equilibrated with Buffer D. The column was then washed with 30 ml of Buffer D and was then eluted with 150 ml of a linear KCl gradient (0.1 M → 0.3 M KCl) in Buffer D. Approximately 3 ml fractions were collected. The fractions were then dialyzed against Buffer D containing 0.1 M KCl. The fractions were

Table I

Purification of EIF-1 from Rabbit Reticulocytes

Fractions	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	units[a]	units/mg	fold	%
Fraction I 0.5 M KCl Ribosomal wash	1,039	19,741	19	1	100
Fraction II DEAE-cellulose batch elution 0-80% $(NH_4)_2SO_4$ concentration	104.5	10,972	105	5.5	55
Fraction III DEAE-cellulose gradient elution	11.3	3,158	319	16.9	16
Fraction IV Hydroxyapatite	0.67	1,350	2,000	105	6.6

[a]One unit of activity is defined as that amount of protein required to bind 1 pmole [^{35}S]Met-tRNA$_f^{Met}$ (in the presence of GTP) under the assay conditions.

then concentrated to approximately 0.5 ml by dialysis against 20% polyetheylene glycol in Buffer D. The concentrated fractions were again dialyzed against Buffer D for 6 hours (Fraction III). The dialyzed Fraction III was then passed through a hydroxyapatite column (1 cm x 5 cm) equilibrated with Buffer E (10 mM potassium phosphate buffer pH 7.5, 1 mM dithiothreitol, 10 percent glycerol). The column was then washed with 0.2 M potassium phosphate buffer, pH 7.5. The proteins were then eluted from the column using a potassium phosphate (pH 7.5) gradient (0.2 M → 0.5 M) containing 1 mM dithiothreitol and 10% glycerol.

Table I summarizes the purification of EIF-1 from crude 0.5 M KCl ribosomal wash. The Fraction IV preparation is approximately 105 fold enriched over the crude ribosomal salt wash and binds 2 nmoles Met-tRNA$_f^{Met}$ per mg protein. This preparation is extremely unstable in dilute solution. The fraction as it elutes from the hydroxyapatite column is dialyzed for 2-3 hours to remove phosphate buffer and is immediately concentrated by passage through a small DEAE-cellulose column and one step elution with 0.3 M KCl. During this concentration, the specific activity of EIF-1 is reduced to approximately 40-50 percent. However,

Table II

$[^{35}S]$Met-$tRNA_f^{Met}$ and $[^{3}H]$Poly r(A) Binding Activities of the EIF-1 Fractions at Different Stages of Purification

Fractions	Amounts Added (µg)	Radioactivity Bound to Millipore Filters (cpm)			Ratio $\frac{[^{35}S]\text{Met-}tRNA_f^{Met}(+GTP)}{[^{3}H]\text{ Poly r(A)}}$
		Retic. $[^{35}S]$Met-$tRNA_f^{Met}$		$[^{3}H]$Poly r(A)	
		-GTP	+GTP		
Fraction I	100	8,400	34,910	7,170	4.9
Fraction II	19	4,270	36,900	2,630	14
Fraction III	9.5	3,420	36,310	1,830	20
Fraction IV	2.5	2,700	44,800	720	62

Standard Millipore filtration assays as described under Materials and Methods were used.

the concentrated preparation is relatively stable at the ice bath temperature and can be stored in liquid nitrogen without appreciable loss in activity over a 2-3 month period. The experiments described in this paper were done using concentrated Fraction IV EIF-1 preparation.

Characteristics of binding of EIF-1 to $[^{35}S]$Met-$tRNA_f^{Met}$ and $[^{3}H]$ Poly r(A).

The characteristics of binding of EIF-1 fractions at different stages of purification were studied using $[^{35}S]$Met-$tRNA_f^{Met}$ and also $[^{3}H]$ poly r(A) messenger (Table II). As expected, all the fractions bound Met-$tRNA_f^{Met}$ and such binding was GTP dependent. GTP dependence of Met-$tRNA_f^{Met}$ binding increased significantly as the fractions were purified. Fraction IV gave 16 fold stimulation of Met-$tRNA_f^{Met}$ binding upon addition of GTP. These fractions were also studied for poly r(A) binding activity. Fraction I preparation showed considerable poly r(A) binding activity and this activity was reduced as EIF-1 was purified. The ratio of GTP dependent Met-$tRNA_f^{Met}$ binding to poly r-A binding activity increased from 4.9 in crude preparation to 62 in Fraction IV. In Table II, the binding of these fractions to $[^{3}H]$ poly r(A) messenger has been described. A similar binding pattern was also observed when other RNAs such as $[^{3}H]$ poly r(U) or E. coli $[^{35}S]$fMet-$tRNA_f^{Met}$ (EIF-1 does not bind E. coli fMet-

TABLE III

Effects of Additions of Crude Retic.tRNA on RNA Binding Activities of EIF-1 Preparations at Different Stages of Purification

Fraction	Amounts Added (μg)	Crude Retic. tRNA Added (μg)	Radioactivity Bound to Millipore Filters (cpm)	
			Retic. [^{35}S]Met-tRNA$_f^{Met}$	[^{3}H] Poly r(A)
Fraction I	100	None	36,460	7,380
		2	36,620	4,960
		10	35,570	3,450
Fraction II	20	None	35,570	2,130
		2	35,130	1,330
		10	34,030	870
Fraction III	9	None	40,160	1,270
		2	39,310	560
		10	38,030	380
Fraction IV	2.5	None	40,900	690
		2	41,040	300
		5	39,400	210
		10	41,080	--
		25	39,200	--
		50	34,890	--

Standard Millipore filtration assay methods were used.

tRNA$_f^{Met}$ (12-13)) were used. The results presented in Table III also confirm that this poly r(A) binding factor is unspecific; addition of crude reticulocyte tRNA competitively inhibits [^{3}H] poly r(A) binding. On the other hand, the binding of EIF-1 is specific for charged Met-tRNA$_f^{Met}$; addition of several fold excess crude uncharged tRNA does not appreciably alter such binding activity.

The bulk of the poly r(A) binding protein is removed during purification of EIF-1 by DEAE-cellulose chromatography (Table IV). This fraction is eluted from the DEAE-cellulose column with Buffer D containing 0.1 M KCl (Fraction IIA). Under these conditions EIF-1 activity is retained in the column and is eluted with buffer containing 0.3 M KCl (Fraction IIB). Fraction IIA contains 55 percent poly r(A) binding activity and insignificant EIF-1 activity. Fraction IIB contains most of the EIF-1 activity and 39 percent poly r(A) binding protein.

TABLE IV

Resolution of the Met-tRNA$_f^{Met}$ Binding Activity (EIF-1) from the Unspecific RNA Binding Protein by DEAE-cellulose Chromatography

Fractions	Total Volume (ml)	Amounts Added per Assay (µl)	Radioactivity Bound to Millipore Filters (cpm)				
			Retic. [^{35}S]Met-tRNA$_f^{Met}$		Percent Recovery	[^{3}H]Poly r(A)	Percent Recovery
			-GTP	+GTP			
Fraction I	23	5	7,670	29,460	100	5,110	100
Fraction IIA 0.1 M KCl elution	27	5	490	620	2	2,340	55
Fraction IIB 0.3 M KCl eluent	26	5	1,580	15,770	59	1,810	39

Standard Millipore filtration assay methods were used.

DISCUSSION

The results presented in this paper clearly establish that EIF-1 binds specifically to Met-tRNA$_f^{Met}$ and that such binding is strongly GTP dependent. On the other hand, crude ribosomal salt wash contains protein factor(s) which bind unspecifically to different RNAs including poly r(A). We have previously reported (3) the presence of one such RNA binding protein, Fx in the high salt wash of reticulocyte ribosomes. This factor binds to different RNA species, although with varying efficiencies; binding to messenger RNAs was more efficient than binding to transfer RNAs. In protein synthesis experiments, Fx was found to be strongly inhibitory. The relationship of the poly r(A) binding protein factor described in this paper to Fx is not clear, at present. Preliminary studies indicate that these two proteins may be different. For example, Fx is heat stable whereas RNA binding protein that is associated with Fraction III and Fraction IV EIF-1 preparations is heat labile. Further studies will be necessary to determine if this RNA binding protein has any role in protein synthesis. Several unspecific RNA binding proteins are also present in E. coli ribosomal high salt wash (16-17). However, the E. coli peptide chain

initiation factor IF-2, like reticulocyte EIF-1, binds specifically to its initiator tRNA; fMet-$tRNA_f^{Met}$, in the case of E. coli (16-17).

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