POLYPEPTIDE SYNTHESIS INHIBITION BY A FACTOR INDUCING STABILIZATION OF 30 S–50 S RIBOSOMAL COUPLES

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1. Introduction

Previous reports from our laboratory have shown that a high salt ribosomal wash fraction obtained from *Bacillus stearothermophilus* is able to induce the in vitro association of ribosomal subunits at low magnesium concentration [1–3]. This effect is due to the action of an association system containing polyamines (AF_{II}) and a protein component (AF_I) [3]. AF_{II} is able to provoke the temperature-dependent association of ribosomal subunits to form 30 S–50 S couples which appear as a broad peak of about 67 S in the sucrose gradient profiles [3]. On the other hand, AF_I causes the stabilization of ribosomal couples at 0°C, in such a way that treated 30 S–50 S couples sediment as a neat peak of 70 S when submitted to sucrose gradient analysis [3].

AF_I could be similar to the coupling factor found [4] in *E. coli*.

Since ribosomes undergo dissociation, association and conformational changes during translation it could be expected that the stability of the 30 S–50 S couples would modify the polypeptide synthetic capacity of ribosomal particles.

We report here the purification of the AF_I and its inhibitory effect on polyphenylalanine synthesis.

2. Materials and methods

Poly(U) as K^+ salt was purchased from Sigma; *E. coli* W stripped tRNA from General Biochemicals; [^{14}C]phenylalanine from New England; hydroxylapatite (Bio-Gel HTP) from Bio-Rad.

2.1. *Ribosomal subunits purification*

Bacillus stearothermophilus 1503-4R was grown, cells were harvested and ribosomal pellets were obtained as in [5]. The ribosomes were resuspended in a small volume of buffer A (10 mM Tris–HCl, pH 7.8, 5 mM magnesium acetate, 50 mM KCl and 2 mM 2-mercaptoethanol) and then dissociated by increasing KCl concentration to 0.5 M. Subunits 30 S and 50 S were purified by centrifugation during 5 h at 35 000 rev./min in a linear 10–30% sucrose gradient made up in 20 mM Tris–HCl, pH 7.8, 10 mM magensium acetate and 1 M KCl. The fractions corresponding to both subunits were pooled and dialyzed overnight against buffer A. When necessary, they were concentrated by ultrafiltration through a PM 30 Diaflo ultrafiltration membrane. The ribosomal subunits obtained show a strong tendency to spontaneous association when incubated together.

2.2. *Purification of association factor I*

The high salt ribosomal wash was prepared from stationary phase cells as in [1], but omitting the heating step. After centrifugation for 15 h at 100 000 × *g*, AF_I was purified from the supernatant fluid as indicated in table 1. The active fraction emerged from hydroxylapatite was dialyzed against 10 mM Tris–HCl, pH 7.8, 500 mM KCl and 2 mM 2-mercaptoethanol, and then adjusted to 5 mM magnesium acetate.

2.3. *In vitro synthesis of polypeptides*

Polyphenylalanine synthesis was carried out in final vol. 0.25 ml lacking energy regenerating system [6] and containing 75 μg poly(U), 160 μg *E. coli* W

Table 1
Purification of AF_I

Fraction	Total units	Spec. act.	Yield (%)	Purification
Ribosomal wash	40 000	200	100	1
Ammonium sulfate precipitate	40 000	322	100	1.6
CM–Sephadex C-50	3992	2770	9.9	13.6
Hydroxylapatite	1900	4271	4.7	21.4

The assay of stabilization of ribosomal couples was carried out as described in section 2

stripped tRNA, 10 μM [^{14}C]phenylalanine (spec. act. 40 Ci/mol), 40 mM glycylglycine, pH 7, 1.5 mM dithiothreitol, 30 mM NH_4Cl, 45 mM KCl, 3.5 mM ATP, 0.6 mM GTP, 140 μg of a pH 5 enzyme preparation and S-100 fraction precipitated between 45–60% ammonium sulphate saturation [7]. Ribosomes were used as indicated in each case and magnesium acetate concentration was adjusted to 23 mM or 14 mM when the assays were carried out at 60°C or 25°C, respectively.

2.4. *AF_I standard assay*

Reaction mixtures (200 μl) containing 10 mM Tris–HCl, pH 7.8, 5 mM magnesium acetate, 75 mM KCl, 2.5 mM dithiothreitol, 0.4 A_{260} units of 30 S and 50 S particles (ratio 1:2) and AF_I, were incubated at 4°C. Aliquots (150 μl) were layered on linear 5–20% sucrose gradients made up in 20 mM Tris–HCl, pH 7.8, 10 mM magnesium acetate and 50 mM KCl and centrifuged for 100 min at 35 000 rev./min in a SW40 rotor at 4°C.

One unit of stabilizing activity is defined as the amount of AF_I necessary to induce the change of the sedimentation coefficient of 30 S–50 S couples from 67 S to 70 S, as shown in fig.2, using 0.4 A_{260} units of ribosomal particles.

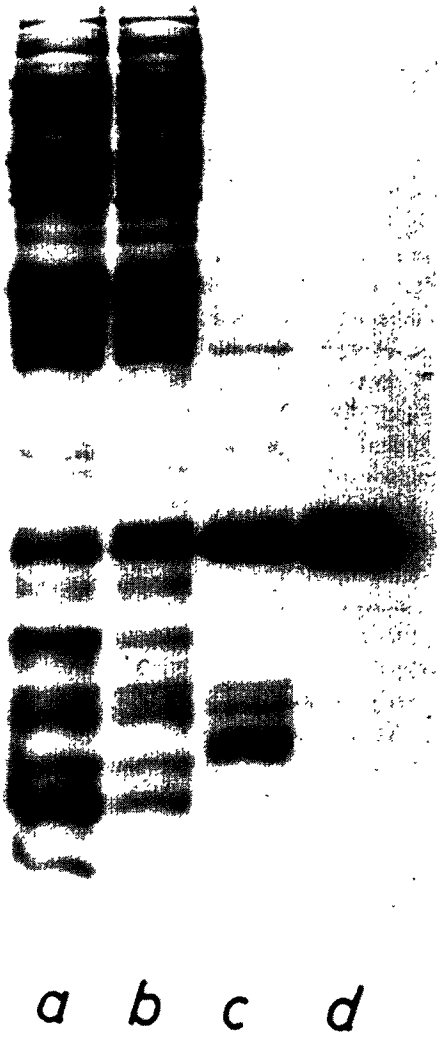

Fig.1. Analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis of AF_I preparations at different steps of the purification procedure. Active fractions were precipitated with 15% trichloroacetic acid, treated and analyzed as in [9] in slab gels (15.5% acrylamide) containing sodium dodecyl sulphate. (a) 75 μg crude ribosomal wash; (b) 45 μg AF_I after precipitation with ammonium sulphate; (c) 10 μg AF_I eluted from CM–Sephadex column; (d) 10 μg factor eluted from hydroxylapatite column.

When the effect of AF_I on protein synthesis was studied the ribosomal subunits (0.9 A_{260} in 200 μl) were preincubated with AF_I at 4°C during the periods indicated in each case; 150 μl were taken and assayed for polyphenylalanine formation.

3. Results and discussion

Table 1 summarizes the purification scheme of AF_I fraction. After chromatography on a hydroxylapatite column a more than 90% pure preparation can be obtained as shown in fig.1, slot d. This preparation provokes the same kind of stabilization of ribosomal couples induced by crude ribosomal wash (fig.2). When the purified fraction was analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis a protein band of 19 500 daltons was obtained.

AF_I is able to inhibit poly(U)-directed polypeptide synthesis. Figure 3 shows the time course of polyphenylalanine synthesis in the presence or absence of AF_I. This inhibition occurs when ribosomes are preincubated with AF_I at 4°C and subsequently added to the mixture used to measure polypeptide synthesis. When translation has already started at 60°C, the addition of AF_I has no inhibitory effect. This result is not due to thermolability of purified AF_I, because very low or no inhibition was observed when the factor was added at 4°C during 5 min to the complete system and poly(U)-directed [^{14}C]phenylalanine incorporation was then assayed at 60°C. The disappearance of the AF_I inhibitory effects on polyphenylalanine synthesis could be due to the formation

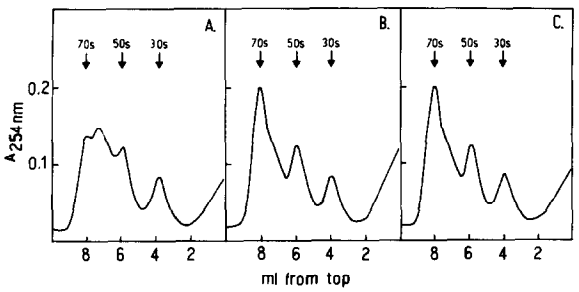

Fig.2. AF_I activity on ribosomal couples. Ribosomal subunits were incubated as described in section 2 with buffer alone (A), with 5 μg crude ribosomal wash (B), or with 0.23 μg AF_I eluted from the hydroxylapatite column (C).

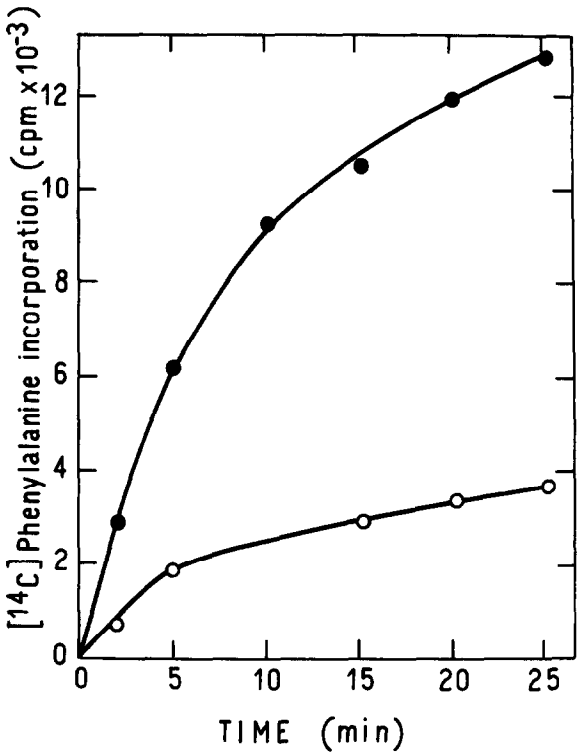

Fig.3. Time course of polyphenylalanine synthesis. Ribosomal subunits were preincubated at 4°C during 30 min and then protein synthesis was assayed at 60°C during the indicated periods of time. Symbols: preincubation carried out without AF_I (•); preincubation of 0.9 A_{260} units of ribosomes with 0.70 μg purified AF_I (○).

at low temperature of a type of initiation complex, which would compete with AF_I added subsequently. Several components of the protein synthesizing system which compete with AF_I in the presence of ribosomes have been detected recently (in preparation).

The level of inhibition of polypeptide synthesis by AF_I seems to be independent of the incubation time during translation (fig.3). Studies on the effect of the preincubation period of ribosomes with AF_I have shown that the inhibition of polyphenylalanine synthesis remained unchanged from 2–30 min of preincubation. On the other hand, the optimum magnesium concentration of the polypeptide synthesis is the same either in the absence or presence of AF_I (23 mM and 14 mM magnesium at 60°C and 25°C, respectively). The factor does not affect amino acid activation.

Table 2
Effect of high salt washing on AF_I treated ribosomes

AF_I during preincubation	K^+ in washing buffer	Polypeptide synthesis (cpm/A_{260} unit or ribosomes)	Inhibition (%)
–	Direct assay	20 036	
+	Direct assay	2497	88
–	0.05 M	22 304	
+	0.05 M	2778	87.5
–	1 M	20 359	
+	1 M	14 061	30.5

Ribosomes (10 A_{260} units/ml) were incubated with 40 μg/ml of a partially purified AF_I preparation during 30 min at 4°C. After dilution with an equal volume of either buffer A or a Buffer containing 10 mM Tris–HCl, pH 7.8, 10 mM magnesium acetate, 1 M KCl and 2 mM 2-mercaptoethanol, the mixture was centrifuged at 150 000 × g over 5 h. The pellets were resuspended in 200 μl of buffer A and used for polyphenylalanine synthesis assays carried out at 60°C over 12 min

The purified factor lacks ribonuclease and proteolytic activities (assayed both with poly(U) and ribosomes). However it could be argued that AF_I produces some minor degradative changes on ribosomal particles which are not detected by our methods. This possibility has been discarded by the results shown in table 2. Polyphenylalanine was inhibited 88% when ribosomes preincubated with AF_I were used. The treated ribosomal particles showed the same low activity after sedimentation in a medium containing 50 mM KCl, indicating that AF_I is still bound to the ribosomes. On the other hand, when treated ribosomes were washed with a high salt containing buffer the inhibition decreased markedly and 70% of the original activity was recovered. This result strongly suggests that AF_I does not produce an irreversible damage of ribosomes.

Figure 4 shows the inhibition of both protein synthesis and aminoacyl-tRNA binding [8] at different concentrations of AF_I. The resulting inhibition is similar for both processes measured at 25°C, suggesting that the inhibitory effect of AF_I on polypeptide synthesis is due to a decrease of the aminoacyl-tRNA binding to ribosomes. The maximum binding was reached at 10 mM magnesium, without increasing with higher concentrations, both in the absence or presence of AF_I.

The factor lacks deacylase activity. The cold

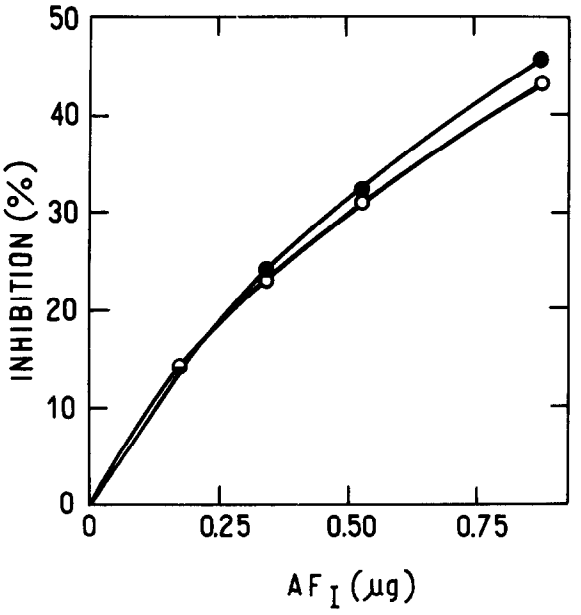

Fig.4. Effect of AF_I concentration on polyphenylalanine synthesis and [^{14}C]Phe-tRNA binding to ribosomes. Ribosomes (1.8 A_{260} units) were preincubated at 4°C with the indicated amounts of AF_I and then used for polyphenylalanine synthesis and Phe-tRNA binding assays at 25°C during 20 min. Binding was performed with 33 pmol [^{14}C]Phe-tRNA (160 μg tRNA) containing 31 500 cpm. Symbols: (○) polyphenylalanine synthesis (maximum incorporation 1521 cpm); (●) Phe-tRNA binding (maximum binding 4521 cpm).

trichloroacetic acid precipitable radioactivity values were identical in reaction mixtures containing untreated or AF_I-treated ribosomes, whereas at the same time a 61% inhibition of binding was observed with AF_I-treated ribosomes.

The stabilization of ribosomal couples, the inhibition of polypeptide synthesis and the decrease of the poly(U)·Phe-tRNA·ribosome complex formation induced by AF_I, together with the lack of action after protein synthesis has already started, strongly suggest that the inhibitory effect occurs at the initiation level of translation.

Other experiments to be described elsewhere demonstrate that AF_I is responsible for the low levels of polypeptide synthesis displayed by ribosomes obtained from stationary phase bacteria.

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