ASSOCIATION OF POLYPEPTIDE INITIATION FACTORS WITH 30 S RIBOSOMAL SUBUNITS

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1. Introduction

Unequivocal evidence that protein factors required for translation of natural messengers in cell-free *E. coli* systems are associated with ribosomes and concerned with chain initiation was first obtained in this laboratory [1]. Subsequent work showed that the factors function in the messenger-directed binding of formylmethionyl-transfer RNA_F (Fmet~$tRNA_F$) to ribosomes [2] and that the binding involves the 30 S ribosomes [3,4], suggesting that the initiation factors are derived from the 30 S subunits. Using the translation of phage RNA by a cell-free *E. coli* system as an assay for initiation factors we have found that whereas no factor activity is obtained from NH_4Cl washes of 50 S ribosomes all of the activity derived from 70 S ribosomes can be recovered from the 30 S subunits. The 30 S origin of the factors was also indicated by hybridization of NH_4Cl-washed and unwashed 50 S and 30 S subunits.

2. Methods

E. coli Q13 ribosomes were washed with a buffer containing 1.0 M NH_4Cl, 20 mM Tris-HCl, pH 7.8, 10 mM magnesium acetate, 1 mM dithiothreitol (DTT), and purified by DEAE-cellulose chromatography as previously described [5]. Isolation of 50 S and 30 S subunits was carried out by centrifugation through 30 ml of a linear 5–30% sucrose gradient in a buffer containing 0.5 M NH_4Cl, 20 mM Tris-HCl, pH 7.8, 2 mM magnesium acetate, and 1 mM DTT. Gradients were equilibrated for 5 hours at 5°, loaded with 0.5 ml of sample containing 160 A_{260} units of purified ribosomes and centrifuged at 23,000 rpm and 4° in the SW 25.1 rotor of the Spinco Model L centrifuge for 12.5 hours. Fractions comprising the faster sedimenting 14% of the 50 S peak and the slower sedimenting 60% of the 30 S peak were pooled. The Mg^{2+} concentration of the fractions was raised to 12 mM and the subunits concentrated by centrifugation in the Spinco 65 rotor for 10 hours at 63,000 rpm and 4°. The pellets were suspended in a buffer containing 0.5 M NH_4Cl, 20 mM Tris-HCl, pH 7.8, 10 mM magnesium acetate, and 1 mM DTT, and stored at 4°.

Unwashed ribosomes were prepared as follows. All operations were conducted at 5° unless otherwise stated. Freshly grown *E. coli* Q13 cells (47 g) were washed once with 50 mM NH_4Cl, 20 mM Tris-HCl, pH 7.8, 10 mM magnesium acetate, and 0.5 mM DTT (buffer A). The washed cells were ground with 94 g of alumina (Fisher, A-542) and extracted with 70 ml of buffer A. Cell fragments and alumina were removed by centrifugation at 15,000 rpm for 30 min in a Servall angle centrifuge. The supernatant was incubated with 3 μg of DNase (Worthington, chromatographically purified) per ml for 10 min at 37° and centrifuged for 30 min at 15,000 rpm. The supernatant (S-30 extract) was centrifuged for 3 hours at 49,000 rpm in the Spinco 50 rotor. The ribosomal pellet was suspended in buffer A and stirred for 2 hours. After removal of debris by centrifugation for 30 min at 15,000 rpm (Servall angle centrifuge) the ribosomes were pelleted once more by centrifugation in the Spinco 50 rotor for 4 hours at 49,000 rpm. The

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washing with buffer A was repeated and the final ribosomal pellet was suspended in a buffer containing 50 mM NH_4Cl, 20 mM Tris-HCl, pH 7.8, 0.1 mM magnesium acetate and 0.5 mM DTT (buffer B) and dialyzed overnight against the same buffer. 50 S and 30 S subunits were prepared as described above for the purified ribosomes except for the use of buffer B in the sucrose gradient and suspension of the pellets in buffer A. They were stored at 4° until used. These subunits were used within 24 hours of preparation because they lose activity much more rapidly than those prepared from NH_4Cl-washed ribosomes.

Crude ammonium sulfate fractions of initiation factors were prepared from 70 S ribosomes as previously described [6]. Similar fractions from unwashed 50 S (66 A_{260} units) and 30 S (125 A_{260} units) subunits were prepared by washing for 12 hours at 5° with a buffer containing 1 M NH_4Cl, 20 mM Tris-HCl, pH 7.8, 10 mM magnesium acetate, and 1 mM DTT. The subunits were pelleted by centrifugation in the Spinco SW 65 rotor for 7 hours at 63,000 rpm and each supernatant was dialyzed overnight against ammonium sulfate at 80% saturation in buffer A. The precipitates were suspended in 0.1 ml of buffer A and dialyzed overnight against the same buffer. The preparations were stored at 4°.

For the amino acid incorporation assay the samples (0.125 ml) contained Tris-HCl, pH 7.8, 50 mM; NH_4Cl, 9 mM; magnesium acetate, 14 mM; 2-mercaptoethanol, 18 mM; ATP, 1.3 mM; GTP, 0.3 mM; phosphocreatine, 17.3 mM; creatine kinase, 3.1 μg; tRNA (*E. coli* W), 125 μg; Q_β RNA, 40 μg; S-150 supernatant fraction (*E. coli* Q13) with 0.3 mg of protein; (^{14}C)lysine (specific radioactivity, 10 μc/μmole), 0.1 mM, the remaining (unlabeled) 19 amino acids, each 0.1 mM; and purified (*E. coli* Q13) ribosomes, 5 A_{260} units, or ribosomal subunits as specified in the legend to table 2. Incubation was for 20 min at 37°. Other details were as previously described [5].

3. Results

It is clear from table 1 that no factor activity was obtained from 50 S ribosomes. On the other hand the NH_4Cl fraction from the 30 S subunits had strong factor activity. In fact, with 15 μg of protein, the activity derived from the 30 S subunits was the same as that contained from the 70 S ribosomes. When fractions from the ammonium chloride wash of 50 S were mixed with those from 30 S subunits (results given in column 2) a slight inhibition was observed. These

Table 1

Association of initiation factors with 30 S ribosomes. 30 S, 50 S, and 70 S ribosomes of *E. coli* Q13 were separately washed with 1.0 M NH_4Cl. Ammonium sulfate fractions were prepared from each wash and assayed for initiation factor activity as described in section 2.

Amount of $(NH_4)_2SO_4$ fraction added (μg protein)	Activity * with $(NH_4)_2SO_4$ fraction from		
	30 S	50 S	70 S
3.0	93	–	–
5.0	–	35	–
7.5	221	–	–
10.0	–	35	–
15.0	373 (295) †	–	373
30.0	332 (264) ††	–	–

* Net [^{14}C]-lysine incorporation (20 min at 37°) in μμmoles/sample (0.125 ml) after subtraction of blank (33 μμmoles) without addition of factors. Values in parentheses (2nd column).

† Factor additions, 15 μg from 30 S + 5 μg from 50 S.

†† 30 μg from 30 S + 10 μg from 50 S.

Table 2

Hybridrization of NH_4Cl-washed and unwashed ribosomal subunits. Either unwashed or NH_4Cl-washed ribosomes of *E. coli* Q13 were separated into 30 S and 50 S components. Appropriate mixtures of 30 S and 50 S subunits were then assayed for polypeptide synthesis with Q_β RNA as messenger, with or without addition of crude initiation factors. The amounts of subunits used (in A_{260} units) were: 50 S unwashed, 1.96; 50 S washed, 2.07; 30 S unwashed, 1.14; 30 S washed, 1.26. Activity is expressed in μμmoles of (^{14}C)lysine incorporated per sample (0.125 ml) after incubation for 20 min at 37°.

Ribosomal subunit		Activity		Stimulation by factors
50 S	30 S	Without factors	With factors *	
Unwashed	Unwashed	56	264	4.7
Washed	Washed	14	1040	74.0
Washed	Unwashed	79	500	6.3
Unwashed	Washed	21	736	35.0

* 175 μg of protein/sample.

results clearly show that the initiation factors are exclusively associated with the 30 S ribosomal subunits.

Assays of mixtures of washed 50 S with unwashed 30 S subunits and conversely without or with addition of initiation factors, shown in table 2, lead to the same conclusion. It will be seen that addition of factors resulted in marked stimulation of activity when either both subunits or only the 30 S subunit were washed with NH_4Cl, but only in marginal stimulation when either both subunits or only the 30 S subunit were unwashed. It may be noted that the unwashed ribosomes were less active than the NH_4Cl-washed ones when saturated with factors. We have noticed that ribosomes, and particularly ribosomal subunits, are much less stable prior to washing with NH_4Cl. This may be due to removal of proteases by the washing procedure.

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