RELEASE OF IF2 FROM NATIVE RIBOSOMES BY DILUTION

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1. Introduction

In *E. coli*, IF2 and other polypeptide chain initiation factors have been shown to be associated with the native 30S ribosomal subunits [1, 2]. These factors are removed by washing the ribosomes with a buffer containing relatively high (0.5–1.0 M) concentrations of NH_4Cl. However, in certain eukaryotic systems, e.g., *Artemia salina* embryos [3], rat liver [4], wheat germ [5], initiation factors, functionally similar to IF2, are found in the post-ribosomal supernatant even though the subcellular fractions are prepared in a buffer containing a relatively low concentration of monovalent cation. In the reticulocyte system under these conditions, there is evidence that the initiation factors are ribosome-associated [6] as well as free in the cytoplasm [7].

The present study shows that much of the IF2 is released when native ribosomes are simply *diluted* in a buffer containing relatively low (0.05 M) concentration of NH_4Cl. These results are consistent with the idea [8] that, as in the case of IF3 [9], the interaction of IF2 with the 30S subunit may involve the equilibrium: IF2 + 30S $\rightleftharpoons$ [IF2–30S].

2. Materials and methods

The pH of all buffers was measured at 25°C. Native ribosomes were prepared as follows: Fresh *E. coli* Q13 cells were washed once with buffer A containing 10 mM magnesium acetate, 20 mM Tris–HCl, pH 7.8, 50 mM NH_4Cl and 0.5 mM dithiothreitol (DTT). The cells were broken by grinding in a mortar with alumina and then suspended in buffer A (2 g of alumina and 1.5 ml of buffer A were used per gram of cells). Cell debris and alumina were removed by centrifugation at 30 000 *g* for 30 min. The supernatant solution was incubated at 37°C for 30 min, cooled in ice, incubated for 5–10 min at 0°C with 3 μg of DNAase per ml and centrifuged again at 30 000 *g* for 30 min. The supernatant (S-30 extract) was centrifuged for 2.5 hr at 60 000 rpm in the Spinco No. 65 ultracentrifuge rotor. The ribosomal pellet was suspended in a minimal volume of buffer A and centrifuged for 30 min at 10 000 rpm in the SS-34 rotor of the Servall centrifuge. The supernatant was used as a source of native ribosomes.

For table 1, experiment 1, three different dilutions of native ribosomes were made. Aliquots of native ribosomes containing a total of 4680, 1944 and 1020 A_{260} units were diluted with buffer A to give a final ribosome concentration of 390, 81 and 17 A_{260} units per ml, respectively. The diluted ribosome samples were then repelleted by centrifugation at 60 000 rpm for 3 hr in the Spinco No. 65 rotor and resuspended in a volume of buffer A approximately equal to the original aliquot *(low-salt ribosomes)*. The supernatant solutions were dialyzed overnight against 80% saturated $(NH_4)_2SO_4$ solution, pH 7.3, containing 0.2 mM dithiothreitol. After centrifugation, the precipitates were taken up in a minimal volume of buffer A without Mg^{2+}, and dialyzed against this buffer for 3–4 hr with several changes of buffer *(supernatant)*. For table 1, experiment 2, native ribosomes were prepared from another batch of Q13 cells. Aliquots containing a total of 4637 and 1003 A_{260} units were diluted to 393 and 17 A_{260} units

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Table 1
Effect of dilution on the release of IF2 from native ribosomes

Expt. no.	Concentration of native ribosomes+ (A_{260} units/ml)	Content of IF2 (a) Supernatant (units)*	(a) Supernatant (sp.act.)**	(b) Low-salt ribosomes (units)*	(c) High-salt ribosomal wash (units)*	(c) High-salt ribosomal wash (sp.act.)**	Total units (a) + (b) or (c)	Percent of total units in supernatant
1	390	798	0.04	13 290	–	–	14 088	6
	81	968	0.07	3541	–	–	4509	21
	17	832	0.12	439	–	–	1271	65
2	393	388	0.02	–	9261	0.90	9649	4
	17	796	0.10	–	383	0.13	1179	68

+ See 'Materials and methods' for details regarding dilution.
* Net values (blanks without IF2 subtracted); 1 unit = 1 pmole of f[^{14}C] Met-tRNA bound to ribosomes.
** Units/μg protein.

The reaction mixtures (0.05 ml) contained: Tris–HCl buffer, pH 7.3, 50 mM; Mg^{2+}, 4 mM; dithiothreitol, 0.5 mM; GTP, 02 mM; NH_4Cl, 90 mM; AUG, 0.06 A_{260} units; f[^{14}C] Met-tRNA, 16 pmoles (360 cpm/pmole). For assay of IF2 activity in the supernatant and high-salt ribosomal wash, the above reaction mixtures were supplemented with 2.0 A_{260} units of a standard preparation of high-salt washed *E.coli* Q13 ribosomes and either 24–95 μg of supernatant protein or 2.9–20 μg of high-salt ribosomal wash. IF2 activity in low-salt ribosomes was assayed by adding 0.5–2.6 A_{260} units of these ribosomes to reaction mixtures. The reaction was started by adding ribosomes and incubation was for 15 min at 0°C. The ribosomal binding of f[^{14}C] Met-tRNA was determined by Millipore filtration.

per ml, respectively, and worked up essentially as described above. The low-salt washed ribosomes were then further washed overnight with a buffer containing 1 M NH_4Cl, 20 mM Tris–HCl, pH 8.1, 5 mM Mg^{2+} and 0.5 mM DTT to extract ribosome-bound initiation factors. The suspension was centrifuged for 3 hr at 60 000 rpm in the Spinco No. 65 rotor. The protein in the supernatant solution was precipitated by dialysis overnight against 80% saturated $(NH_4)_2SO_4$ solution, pH 7.3 containing 0.2 mM DTT. The precipitate was taken up and dialyzed against buffer A without Mg^{2+} as described above *(high-salt ribosome wash).* IF2 activity was assayed by measuring the AUG-directed ribosomal binding of f[^{14}C] Met-tRNA at 0°C as described previously [10]. Protein was measured by the method of Lowry et al. [11].

The supernatant fractions used in the experiments of table 2 were prepared as follows. Native ribosomes (3 ml) were layered on 10–30% linear sucrose gradients (54 ml) in buffer A. The gradients were centrifuged in the Beckman SW 25.2 rotor for about 14 hr at 21 000–22 000 rpm. The gradients were fractionated into plastic tubes containing sufficient buffered NH_4Cl such that the final concentration of NH_4Cl would be about 1 M in each fraction. After analysis of the optical density (A_{260}) profile, appropriate fractions from the *top* of the gradient were pooled and then subjected to ammonium sulfate precipitation and dialysis as described in the preceding paragraph. As judged by analytical sucrose gradient centrifugation, no ribosomal subunits were detectable in the supernatant fractions prepared by this method.

3. Results

It may be seen from table 1 that (a) the specific activity of IF2 in the supernatant fractions increases with increasing dilution of native ribosomes (column 4). (b) The fraction of total IF2 activity recovered in the supernatant fraction also shows a progressive increase as a result of increasing dilution of ribosomes (last column). This is true regardless of whether the total IF2 activity is expressed as the sum of IF2 activities present in the supernatant and low-salt washed

Table 2
Effect of dilution on release of IF2 from native ribosomes

Expt. no.	Concentration of native ribosomes (A_{260} units/ml)	Total amount of native ribosomes (A_{260} units) (a)	Content of IF2		
			Supernatant		
			Total units (b)	Sp.Act.	b/a
1	310	931	115	0.03	0.12
	31	186	61	0.1	0.33
2	387	1162	40	0.01	0.03
	29	174	14	0.03	0.08

IF2 activity in the supernatant fractions was assayed essentially as described in table 1.

ribosomes (expt. 1, columns 3, 5, 8) or as the sum of that present in the supernatant and the high-salt ribosomal wash (expt. 2, columns 3, 6, 8). (c) In contrast, the specific activity of IF2 recovered in the high-salt ribosomal wash is considerably lowered by prior dilution of native ribosomes (expt. 2, column 7), since as mentioned above, more IF2 is released from the ribosomes into the supernatant by lowering the concentration of native ribosomes. It should be pointed out that the recovery of *total* IF2 activity appears to be less, the higher the dilution of native ribosomes. In expt. 1, for example, the yield of *total* IF2 units (per 100 A_{260} units of native ribosomes) is about 300, 230 and 130, respectively for the three dilutions used. The reasons for these lower yields at higher dilutions probably include an increased adsorption of IF2 (and IF3) to glass surfaces [12, 13] and an increased loss of IF1 during dialysis [14].

Analytical sucrose gradient centrifugation revealed that small but detectable amounts of ribosomal subunits were still present as contaminants in these high-speed supernatant fractions. In addition, the level of contaminating subunits was more, the higher the dilution of native ribosomes. This is presumably due to the fact that dilution of 50S–30S couples (which constitute the bulk of native ribosomes) leads to an increased formation of *free* 50S and 30S subunits by shifting the equilibrium: 50S–30S $\rightleftharpoons$ 50S+30S, to the right [15]. However, since 50S–30S couples contain no IF2 or other initiation factors [1,2,16], the effect of dilution on the equilibrium just described should *not* contribute to the amount of IF2 which is recovered in the high-speed supernatant fractions. This is further substantiated by the results summarized in table 2. The supernatants used in these experiments were prepared by a different procedure (see Materials and methods) and did not contain detectable amounts of ribosomal subunits. It may be seen that the IF2 activity (per A_{260} unit of native ribosomes layered on sucrose gradients) recovered in the supernatant fractions again increases with dilution of native ribosomes (column 6, table 2). There is a parallel rise in the specific activity of IF2 also (column 5, table 2). The *total* recovery of IF2 activity from the more highly diluted sample of native ribosomes was, however, very low in these experiments. This is presumably due to the fact that the experiments of table 2 involved prolonged periods of centrifugation (14 hr) as well as increased dilutions (3 ml of native ribosomes layered on 50–55 ml sucrose gradients). It appears likely, therefore, that much of the IF2 which was released into the supernatant by dilution of native ribosomes, was not detected in the experiments of table 2.

5. Discussion

It has recently been proposed [8] that the first step in the IF2-dependent, AUG-directed binding of fMet-tRNA to factor-depleted ribosomes may involve the equilibrium: IF2 + 30S $\rightleftharpoons$ [IF2–30S]. If so, a higher proportion of the total IF2 should exist *free* when the concentration of the [IF2–30S] complex is lowered. The results of the present study

are consistent with this view, since dilution of native ribosomes, which contain bound IF2 (as well as bound IF1 and IF3), in a buffer with relatively low concentration of NH_4Cl, leads to release of IF2 activity in the supernatant. It is likely that the observed distribution of polypeptide chain initiation factors (prokaryotic and eukaryotic) between ribosomes and postribosomal supernatant may reflect the dissociation constants of the respective factor(s)–ribosome complexes as well as the dilution of such complexes during isolation. It would also appear that the dissociation constants of IF2–ribosome complexes are increased in the presence of buffer containing a relatively high concentration of NH_4Cl, since only traces of IF2 activity are present in high-salt washed ribosomes. Finally, it should be mentioned that Fakunding and Hershey [17], using radioactively labelled IF2, have directly demonstrated that this factor binds to the 30S subunit and that this binding is stabilized by IF1 and IF3. Similar results have been obtained in other laboratories [18,19].

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