NATURE OF EUKARYOTIC PROTEINS REQUIRED FOR JOINING OF 40S and 60S RIBOSOMAL SUBUNITS

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SUMMARY

A mixture of 40S and 60S subunits from salt-washed rabbit reticulocyte ribosomes fails to promote methionyl-puromycin synthesis under conditions in which an AUG-40S-Met-tRNA$_i$ initiation complex, but not an 80S complex, is readily formed. This suggests that the inability of the system to form methionyl-puromycin is due to failure of the subunits to join. When Artemia salina 60S subunits are substituted for their reticulocyte counterparts, the resulting hybrid system readily forms an 80S initiation complex and synthesizes methionyl-puromycin. Activity of the reticulocyte 60S subunits can be restored by factors IF-M2A and IF-M2B. This suggests that one or both of these factors may be 60S proteins, essential for subunit joining, that may be removed from ribosomes by salt washing procedures.

The rabbit reticulocyte and Artemia salina initiation factors IF-M1 (1,2) and EIF-1 (3) are functionally identical (4,5). Either factor promotes the AUG-dependent binding of Met-tRNA$_i$[2] to A. salina 40S ribosomal subunits with no requirement for GTP and, upon addition of 60S subunits, methionyl-puromycin (Met-puro) is formed in good yields (4). Similar results are obtained with rat liver ribosomes (6).

In contrast to the above results, complex requirements were reported (7,8) for Met-puro synthesis by salt-washed rabbit reticulocyte ribosomes; these include IF-M2A and IF-M2B (9) besides IF-M1 (see also

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[2]Met-tRNA$_i$ is the abbreviation for initiator methionyl-transfer RNA.

ref. 10). On reinvestigating this problem, we find that a mixture of 40S and 60S subunits from salt-washed rabbit reticulocyte ribosomes fails to promote Met-puro synthesis under conditions in which an AUG-40S-Met-tRNA$_i$ initiation complex, but not an 80S complex, is readily formed in the presence of A. salina EIF-1. The deficiency can be corrected either (a) by substituting A. salina 60S for reticulocyte 60S subunits, or (b) by addition of factors IF-M2A and IF-M2B. Our results suggest that one or both of these factors are essential for subunit joining and may in fact be 60S proteins that can be released from ribosomes by salt-washing.

MATERIALS AND METHODS

Ribosomes and ribosomal subunits - Unfractionated 0.5M KCl-washed rabbit reticulocyte ribosomes (11) prepared as described are a heterogeneous population of 60S subunits, 40S subunits, and 80S monosomes. Reticulocyte 40S and 60S ribosomal subunits are prepared from the unfractionated salt-washed ribosomes by a procedure involving stirring for 30 min at 0° in 0.5M KCl, 2 mM $MgCl_2$, and centrifugation through a 5-20% linear sucrose gradient containing 300 mM KCl, 20 mM Tris-HCl buffer, pH 7.5, 2 mM $MgCl_2$, and 1 mM dithiothreitol (12). The 40S subunits are homogeneous but the 60S subunits contain some material sedimenting at 80S and above. A. salina 40S and 60S ribosomal subunits are prepared from undeveloped embryos as previously described (13).

Assays- The general plan involved the prior formation of the AUG-40S Met-tRNA$_i$ complex at 0°, as promoted by A. salina EIF-1, followed by formation of the 80S complex, upon addition of 60S subunits, and incubation with puromycin. In the standard assay, duplicate samples (in a final volume of 60µl) containing Tris-HCl buffer, pH 7.4, 80 mM; KCl, 150 mM; $Mg(OAc)_2$, 4.5 mM; dithiothreitol (DTT), 2.0 mM; ApUpG (AUG) 0.05 A_{260} unit; A. salina EIF-1, 0.25 µg; crude E. coli [^{14}C]Met-tRNA (about 450 cpm/pmole), 25 pmoles; and A. salina 40S ribosomal subunits, 0.30 A_{260} unit, are first incubated for 30 min at 0° to form the 40S initiation complex (1st incubation). This is followed by the addition of A. salina 60S subunits, 0.64 A_{260} unit, to each duplicate sample whereupon one of them is processed for determination of the ribosomal binding of Met-tRNA while the other receives 75 µg of puromycin and, after further incubation for 60 min at 0° (2nd incubation), is analyzed for Met-puro synthesis. Ribosomal binding of [^{14}C]Met-tRNA or [^{35}S]Met-tRNA$_i$ and Met-puro synthesis are measured as described (13). Rabbit liver [^{35}S]Met-tRNA$_f$ is prepared as described (11). Highly purified preparations of rabbit reticulocyte IF-M2A and IF-M2B (14, 15) were used. The purification procedure will be described in detail elsewhere. Other preparations and methods are as in previous work (5).

RESULTS

As seen in Fig. 1, with A. salina EIF-1 and ribosomal subunits, 80-90 % of the ribosome-bound Met-tRNA is converted to Met-puro in 60 min at

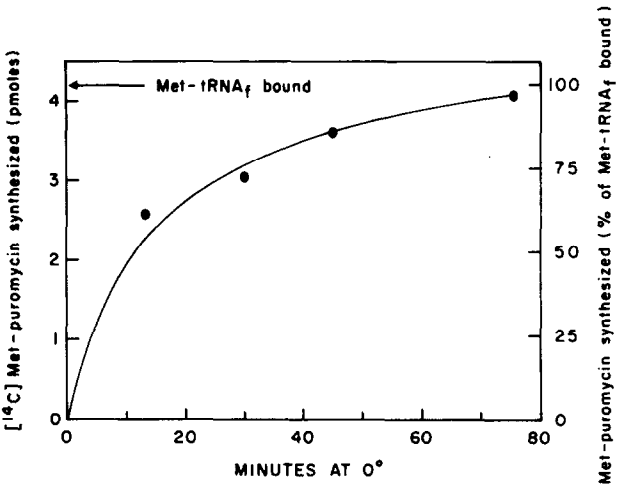

Fig. 1. Time course of Met-puro synthesis at 0° with A. salina ribosomes. Five single standard assay samples were run. One was used for determination of the ribosomal binding of Met-tRNA after adding 60S subunits. The others received puromycin and, after incubation at 0° for various times, were analyzed for [^{14}C]Met-puro synthesis.

0°. These conditions of incubation for Met-puro synthesis were therefore chosen for most experiments. In contrast to these results, no Met-puro is synthesized when reticulocyte subunits are substituted for their A. salina counterparts. This observation could be explained if one or both reticulocyte subunits lacked essential component(s) present in the A. salina subunits. To verify this hypothesis hybrid incubations were conducted with A. salina 40S and reticulocyte 60S or conversely. The results, shown in Table I, are unequivocal; the reticulocyte 60S subunits are inactive. Since peptide bond formation must be preceded by subunit joining, the inactivity of the reticulocyte 60S subunits could be due to inability to join to the 40S initiation complex or, were this not the case, to a deficiency or alteration of a protein(s) essential for peptidyl transfer.

In order to ascertain the nature of the ribosomal lesion, initiation complexes, formed on reticulocyte 40S subunits, were supplemented with either reticulocyte or A. salina 60S subunits and the reaction mixtures analyzed by sucrose density gradient centrifugation. The answer (Fig. 2)

Table 1. AUG- and EIF-1-dependent ribosomal binding of Met-tRNA and Met-puro synthesis with A. salina and rabbit reticulocyte subunits

Sample No.	Source of subunits		[^{14}C]Met-tRNA binding	[^{14}C]Met-puro synthesis	
	40S	60S	(pmoles)	(pmoles)	(% of bound)
1	A. salina	A. salina	4.69	3.91	83
2	A. salina	Reticulocytes	4.47	0.05	1
3	Reticulocytes	Reticulocytes	4.58	0	0
4	Reticulocytes	A. salina	3.87	3.40	88

Four duplicate samples were run as in the standard assay, with the following exceptions. Samples 1 and 2 had A. salina 40S subunits (0.30 A_{260} unit) whereas samples 3 and 4 had reticulocyte 40S subunits (0.35 A_{260} unit) in the first incubation. In the second incubation samples 1 and 4 had A. salina 60S subunits (0.64 A_{260} unit) whereas samples 2 and 3 had reticulocyte 60S subunits (0.70 A_{260} unit). Ribosomal binding of [^{14}C]Met-tRNA and [^{14}C]Met-puro synthesis were determined as described in MATERIALS AND METHODS. All values were corrected for blanks (0.3-0.5 pmole) in the absence of EIF-1.

is again unequivocal; a stable 80S complex is formed only in the samples having A. salina 60S subunits (panel C). These results are consistent with the notion that the 60S subunits derived from salt-washed reticulocyte ribosomes are unable to function in Met-puro synthesis because of their inability to join to the 40S initiation complex. As already mentioned, a requirement of factors (IF-M1, IF-M2A, and IF-M2B) had been reported (7,8) for Met-puro synthesis by salt-washed reticulocyte ribosomes. It is evident from Table II that IF-M2A and IF-M2B, but neither factor alone, restore Met-puro synthesis to the level obtained in their absence when A. salina 60S subunits replace their reticulocyte counterparts.

DISCUSSION

In this paper we show that 60S subunits from salt-washed reticulocyte ribosomes cannot join to an AUG-40S-Met-tRNA$_i$ initiation complex so that

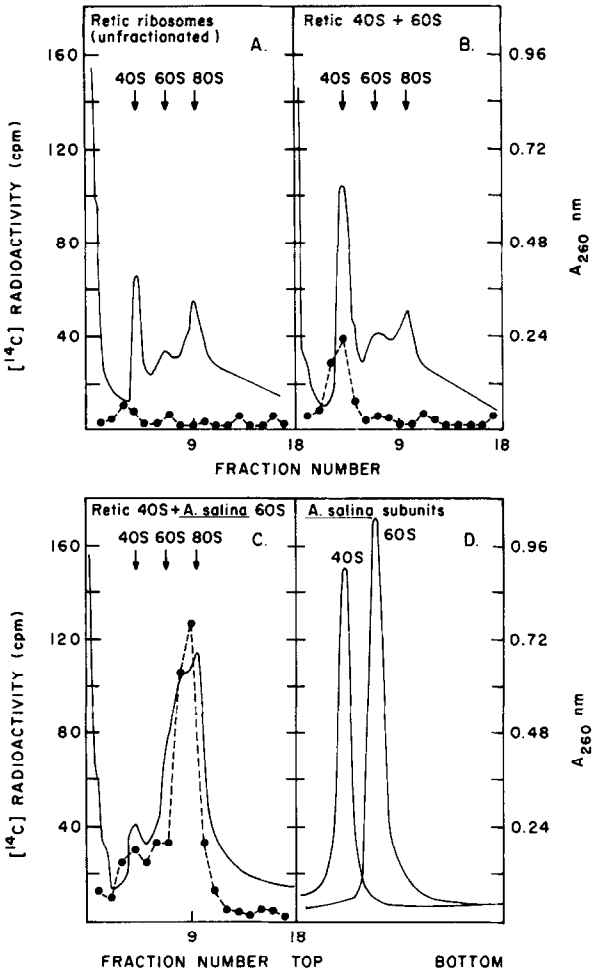

Fig. 2. Subunit joining. Three single samples were run through the first incubation as in the standard assay, except that one (sample A) had unfractionated reticulocyte ribosomes, 1.2 A_{260} units, and two (samples B and C) had reticulocyte 40S, 0.35 A_{260} unit, instead of A. salina 40S subunits. After adding reticulocyte 60S subunits (0.70 A_{260} unit) to sample B, A. salina 60S subunits (0.64 A_{260} unit) to sample C, and an equal volume of water to sample A, 20 µl aliquots were withdrawn for determination of ribosomal binding of [^{14}C]Met-tRNA. The values were (in pmoles/60 µl sample) 2.7, 5.1, and 3.9 in samples A, B, and C, respectively. The remaining 40 µl of each sample was layered over 5 ml of a 15-25 % linear sucrose gradient in 80 mM Tris-HCl, pH 7.4, 150 mM KCl, 4.5 mM $Mg(OAc)_2$, and centrifuged for 75 min at 4° and 45,000 rpm in the SW 50.1 rotor of the Spinco Model L-2 65B centrifuge. The gradients were analyzed in an Isco (Model D) gradient analyzer. Fractions were collected and filtered through Millipore membranes for measurement of ribosome-bound radioactivity. Panel A, reticulocyte ribosomes (unfractionated); panel B, Reticulocyte 40S and 60S subunits; panel C, reticulocyte 40S and A. salina 60S subunits; panel D, sedimentation pattern of A. salina 40S (0.45 A_{260} unit) and 60S (0.73 A_{260} unit) subunits shown for comparison (composite of separate runs). ——— , A_{254} nm; -●-●-●-, ^{14}C radioactivity.

Table II. Requirements for Met-puro synthesis with reticulocyte ribosomal subunits

Source of 60S subunits	Other additions	[^{35}S]Met-puro synthesis (cpm)
Reticulocytes	None	382
"	IF-M2A (0.97 µg)	766
"	IF-M2B (21 µg)	296
"	IF-M2A (0.97 µg) IF-M2B (21 µg)	3178
A. salina	None	3130

Each of five single samples (40 µl) contained Tris-HCl buffer, pH 7.4, KCl, $Mg(OAc)_2$, DTT, and AUG as in the standard assay and in the same concentrations. They contained, in addition, A. salina EIF-1, 0.23 µg; rabbit liver [^{35}S]Met-tRNA (17,500 cpm/pmole), 5.0 pmoles; and rabbit reticulocyte 40S subunits, 0.21 A_{260} unit. The first incubation (to form 40S initiation complex) was for 20 min at 0°. The first four samples were then supplemented (to a final volume of 0.63 µl) with reticulocyte 60S subunits (0.40 A_{260} unit) whereas the fifth sample, which served as a control, was supplemented with A. salina 60S subunits (0.48 A_{260} unit). Then they all received puromycin (75 µg), with other additions as indicated in the table and, after incubation for 15 min at 30°, the samples were analyzed for [^{35}S]Met-puro synthesis. The final concentrations of KCl and Mg(OAc) were 95 and 2.8 mM, respectively.

the system is unable to synthesize Met-puro. Met-puro synthesis can be restored either by the substitution of A. salina 60S subunits or by the addition of IF-M2A and IF-M2B. While the exact mechanism of restoration is unclear, the ability of IF-M2A and IF-M2B to stimulate Met-puro synthesis implicates these proteins in subunit joining and throws light on their function. The relation, if any, of the GTPase (and ATPase) activity of IF-M2A (8) to this function is unknown.

The isolation from reticulocyte ribosomal washes (16-18) and rat liver supernatant (19) of proteins promoting ribosomal subunit association has been reported. These proteins resemble IF-M2A in molecular weight and

chromatographic properties. Thus, the finding that IF-M2A and IF-M2B are concerned with subunit joining is not surprising. However, their relation to the large ribosomal subunit was unexpected. Curiously, 60S subunits from diabetic rat muscle appear to have the same lesion as their salt-washed reticulocyte counterparts for they exhibit a decreased capacity to form 80S couples (20).

It is possible that so-called subunit joining factors may be 60S ribosomal or associated proteins extractable by washing with salt solutions containing little or no Mg^{2+}, a treatment that causes partial unfolding of the ribosomes. The fact that A. salina 60S subunits restore 80S complex formation and Met-puro synthesis, when substituted for their reticulocyte counterparts, testifies to their structural integrity (cf. Fig. 2, panel D). It may be remembered that A. salina ribosomal subunits are prepared (13) in the presence of 11 mM Mg^{2+}.

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