DIRECT BIOCHEMICAL APPROACH TO THE STRUCTURAL HETEROGENEITY OF 30 S RIBOSOMES FROM *ESCHERICHIA COLI*

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1. Introduction

Structural heterogeneity of purified 30 S ribosomes was demonstrated by Voynow and Kurland [1] several years ago. Stoichiometry measurements indicated that three main classes of proteins exist in the 30 S particle: the fractional ones, which are present in amounts corresponding to 0.5 or fewer copies per ribosome; the unit proteins, which are present in amounts equal or very close to one copy per ribosome and the marginal ones, with intermediate stoichiometry or for which precise data are not available.

A definite decision on whether the 30 S ribosome is also heterogeneous *in vivo* cannot be made in the present state of knowledge, neither is it definitely proved that the structural heterogeneity found *in vitro* is not an artifact due to the experimental procedures.

One of the most attractive hypotheses to interpret the structural heterogeneity supposes that the 30 S subunit cycles through different states during protein synthesis, the idea being that there is only one class of 30 S ribosomes which undergo structural changes during the initiation, elongation and termination processes [2, 3]. An alternative explanation suggests, on the contrary, that the composition of 30 S ribosomes does not change during protein synthesis; in this case, one may imagine that the fractional proteins either are unimportant ones, or serve to differentiate ribosomes into different classes with different specificities or regulatory properties.

As suggested previously, the use of matrix bound messenger RNA provides a direct biochemical assay for the possible functional heterogeneity of 30 S ribosomes *in vitro* [4]. This procedure enables the isolation of a specific complex containing 30 S ribosomes engaged in the initiation process, thus presumably homogeneous with respect to their functional state.

In the present work, we apply this technique to the isolation of such initiation competent ribosomes on a preparative scale, and we analyse their ribosomal protein content using total proteins from untreated 30 S ribosomes as reference..

The results show that three proteins (S1, S2 and one unassigned species) are absent in the 30 S particles engaged in the initiation complex, while no significant variation is observed in the stoichiometry of the other ones. The possible release of proteins S1 and S2 during the initiation process is discussed.

2. Experimental

Buffers: Buffer 1: Tris-HCl 10 mM, pH 7.8, Mg acetate 8 mM, mercaptoethanol 6 mM, NH_4Cl 30 mM. Buffer 2: Tris-HCl 10 mM, pH 7.8, EDTA 15 mM, mercaptoethanol 6 mM, NH_4Cl 30 mM. Buffer 3: Tris-HCl 10 mM, pH 7.8, Mg acetate 0.1 mM, mercaptoethanol 6 mM, NH_4Cl 30 mM. Buffer 4: methylamine-acetic acid buffer 30 mM, pH 5.6, urea 6 M, mercaptoethanol 6 mM.

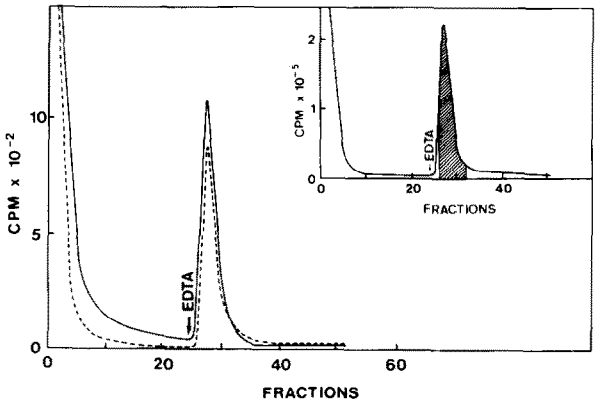

Fig. 1. Isolation of the initiation complex on resin bound poly (A, G, U). The initiation complex was formed in a polyallomer tube by 10 min incubation at 37° in a final volume of 0.5 ml containing 10 mM Tris-HCl buffer, pH 7.8, 8 mM Mg acetate, 30 mM NH_4Cl, 2 mM GTP, 6 mM mercaptoethanol, 350 μg crude initiation factors, 250 μg fMet-tRNA ([^{3}H] Met), 600 μg 30 S ribosomes (^{14}C) and 400 μg poly (A, G, U) supplied as resin coupled derivative. After chilling, the sample was transferred into a small column, washed at 4° with buffer 1, then eluted with buffer 2. (——) fmet-tRNA ([^{3}H] Met) net counts, (- - - -) 30 S (^{14}C) counts. Arrow indicate buffer change. *Insert*: preparative assay. Each component of the above mixture was increased 6-fold, but in this case, unlabelled fMet-tRNA and 8 μCi 30 S ribosomes (^{14}C) were used. Fractions corresponding to hatched area were pooled. (——) 30 S (^{14}C) counts.

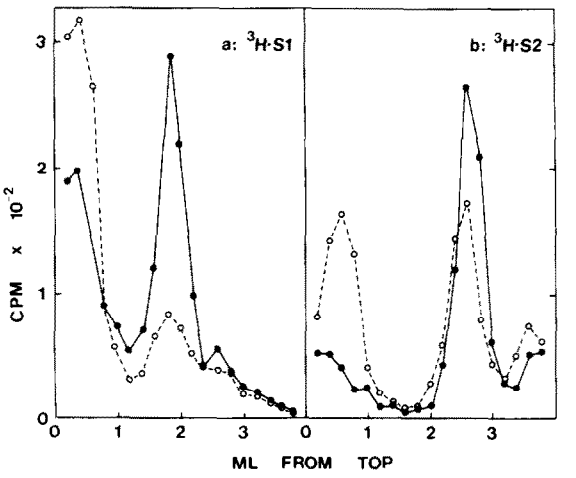

Fig. 2. Release of proteins S1 and S2 from ribosomes engaged in the initiation complex. Ribosomes were labelled only with protein S1 or S2 [3], incubated and analyzed on 10–35% sucrose gradients in buffer 1. 0.2 ml aliquots were collected and counted in Triton emulsion. (a) (●—●—●) 240 μg 30 S ([^{3}H] S1) incubated in buffer 1 for 10 min at 37°. (○—○—○) 240 μg 30 S ([^{3}H] S1) engaged in an initiation complex (reaction mixture as in fig. 1, but poly (A, G, U) was supplied as the free polymer). (b) The same as in (a) but using 30 S ([^{3}H] S2). (●—●—●) Ribosomes incubated alone. (○ - - ○ - - ○) Complete system. The ability of exchange labelled 30 S particles to initiate was checked on Millipore filters before use.

3. Results

Escherichia coli (strain B) was grown in minimal medium supplemented with labelled amino-acids (^{14}C or ^{3}H, NEN Chemicals GmbH) and harvested in mid-exponential phase. Ribosomes, ribosomal subunits, crude initiation factors and fMet-tRNA were prepared as described previously [4]. The coupling of oxidized poly (A,G,U) to the resin and its use to program the formation of the initiation complex were carried out under the conditions already described [4], but on a larger scale.

After 10 min incubation at 37°, the initiation complex was transferred into a small column, washed with buffer 1, then eluted with buffer 2 (15 mM EDTA) or with buffer 3 (0.1 mM Mg^{2+}).

Since in the preparative experiment, we used unlabelled fMet-tRNA, we performed a separate small scale assay with [^{3}H] fMet-tRNA to ascertain that the formation of the initiation complex proceeded normally (fig. 1). 8 μCi of 30 S ribosomes (^{14}C) were engaged in the preparative assay and roughly 6% of this amount was recovered in the EDTA eluate as part of the initiation complex (fig. 1, upper right). The pooled fractions were mixed with reference 30 S (^{3}H) particles to obtain a ^{3}H/^{14}C counting ratio of 2, and with unlabelled carrier 30 S ribosomes (10 mg). The mixture was dialyzed overnight against buffer 4, then digested with T1- and pancreatic-ribonuclease (1:100 w/w of each) [5]. The sample was then applied onto a CM-cellulose column equilibrated in buffer 4, washed, then fractionated in 0.65 ml aliquots using a linear salt gradient (0–0.4 M sodium acetate) [6]. 0.25 ml aliquots were counted in Omnifluor:toluene:Triton emulsion.

The control column was carried out under similar conditions, using the 30 S (^{14}C) fraction which did

Table 1
Protein distribution in 30 S ribosomes engaged in the initiation complex programmed by resin bound poly (A, G, U).

Protein code	$(^{14}C/^{3}H)_{Sx}/(^{14}C/^{3}H)_{S7}$		$\frac{(^{14}C)^{initiating}_{Sx}}{(^{14}C)^{control}_{Sx}}$
Sx	Control 30 S	Initiating 30 S	
S1	0.86	0.13	0.15
S2	0.96	0.19	0.19
S3	0.92	0.84	0.91
S4	1.04	1.00	0.96
S5	0.76	0.67	0.88
S6	1.02	1.05	1.03
S7	1.00	1.00	1.00
S8	1.06	0.89	0.84
S9	0.98	1.10	1.12
S10	0.90	1.07	1.19
S11	1.14	1.12	0.98
(S12-S13)	–	0.99	–
(S14)	–	0.76	–
(S15-S16-S17)	1.10	0.50	0.45
S18	1.00	1.13	1.13
S19	0.98	1.06	1.08
S20	0.84	0.94	1.12
S21	0.90	0.75	0.83

Experimental details are in the text. $^{14}C/^{3}H$ ratios for all proteins were estimated following CM-cellulose chromatography and polyacrylamide gel electrophoresis. The $^{14}C/^{3}H$ values obtained for each Sx protein are divided by the $^{14}C/^{3}H$ ratio measured for protein S7 taken as reference. The third column of the table represents the ratio of the ^{14}C label in each protein of initiating ribosomes to the ^{14}C label of the corresponding species in control particles. Consequently, for any protein with unchanged stoichiometry in the two fractions recovered from the column, this value should remain equal or close to 1. Values lower or higher than 1 indicate respectively the depletion or the enrichement of ribosomes in the protein considered.

not engage in the initiation complex (this fraction passes through the column during the washing with buffer 1).

The $^{14}C/^{3}H$ ratio was monitored and compared for both chromatographies; to identify the proteins eluted from the CM-cellulose column, an aliquot of each peak was submitted to polyacrylamide gel electrophoresis (methylene-bis-acrylamide was replaced by ethylene diacrylate). After staining with Coomassie blue, the bands were sliced, solubilized with NCS (Amersham) and counted to measure the $^{14}C/^{3}H$ ratio.

Table 1 summarizes the data obtained by the combination of the two methods; the values are expressed with respect to the protein S7, a well known unit protein [1] which elutes as a single homogeneous peak in CM-cellulose chromatography.

As can be seen, for most of the ribosomal proteins considered, there is no significant variation between the amount present in the control 30 S and the amount present in "initiating" ribosomes. However, in three cases, namely S1, S2 and one unassigned protein (S15, S16, S17?), there is a drastic decrease in the amount present in the "initiating" particles.

S1 and S2 are clearly fractional proteins [1], but no indications are available for the unassigned species; we were unable to separate the three proteins S15-S16-S17 either on CM-cellulose or by gel electrophoresis.

No precise $^{14}C/^{3}H$ ratio measurements could be obtained for proteins S14 and for the pair S12–S13 because of technical difficulties in recovering the species from the gels. However, the $^{14}C/^{3}H$ ratio for these proteins are identical, as judged by the CM-cellulose elution profiles; therefore, we suspect that their relative amount remains unaffected in the "initiating' ribosomes.

Thus, the results show that only 3 proteins (two of them being undoubtedly fractional) are missing from the 30 S ribosomes engaged in the initiation complex. In contrast, there is no variation in the stoichiometry of marginal and unit proteins, at least for the species we were able to identify with certainty.

Since S1 and, to a lesser extent S2, may spontaneously dissociate from the 30 S subunits in high ionic strength buffers or by repeated washings [7], we asked whether our procedure itself could be responsible for their absence in "initiating" 30 S particles.

Taking advantage from the fact that these two proteins are readily exchangeable *in vitro* [3], we incubated unlabelled 30 S particles with [^{3}H] S1 or [^{3}H] S2 to recover ribosomes enriched and labelled only in one of these two fractional proteins.

These S1- or S2-labelled ribosomes were then analysed on sucrose gradients either in the free state or after being engaged in the initiation complex. Fig. 2a and 2b show that most of the radioactivity sediments with the 30 S particle incubated alone; however, a significant fraction of this radioactivity is displaced from the particle as soon as it engages in the initiation complex.

Therefore, we conclude that the release of proteins S1 and S2 from the ribosome appears to be significant and independent of the methodology used.

4. Discussion

Our results support the hypothesis that 30 S ribosomes are structurally and functionally heterogeneous *in vitro*. Indeed it appears that ribosomes engaged in the initiation process do not contain all the fractional proteins. Two of the missing species have been identified accurately as proteins S1 and S2, the third one remaining yet unassigned.

We have ruled out the objection that the column procedure we used could be responsible for the removal of these proteins from the ribosomes. Our experiments with 30 S ribosomes labelled only in S1 or S2 favours the idea that the release of these two proteins has some physiological significance. However, we have no data to prove that the 30 S ribosomes are still functional after recovery from the column.

It should be pointed out that protein S1, according to Van Duin and Kurland [3], is required for optimal messenger RNA binding to the ribosome. Therefore, in view of our data, one might speculate that this protein is released from the ribosome after having played its role, and becomes available for a new initiation round.

It seems clear also that protein S2, together with S3 and S14, stimulates the binding of fMet-tRNA to 30 S ribosomes in the presence of messenger RNA [8]. Therefore, the release of S2 from 30 S ribosomes which have completed the initiation process is consistent with a possible cycling of this protein from one ribosome to another with concomitant stimulation of fMet-tRNA binding on ribosomes having proteins S3 and S14.

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