RIBOSOMAL PROTEIN S12 AND 'NON-ENZYMATIC' TRANSLOCATION

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1. Introduction

It has been shown earlier that treatment of purified *Escherichia coli* ribosomes with *para*-chloromercuribenzoate (PCMB) activates their ability for translocation in the absence of EF-G and GTP [1,2]. Thus, in the system consisting of PCMB-treated ribosomes, poly U and [^{14}C]phe-tRNA, the non-enzymatic binding of phe-tRNA and transpeptidation are supplemented by non-enzymatic translocation which creates 'non-enzymatic' elongation resulting in the synthesis of long enough [^{14}C]polyphenylalanine chains. The system is completely polyU-dependent, is inhibited by chloramphenicol, erythromycin, viridogrisein, tetracycline, edeine, thiostreptone, spectinomycin, streptomycin, kanamycin, neomycin and is not inhibited by fusidic acid [2,3].

It has been shown that interaction of PCMB with just the 30 S subparticle is completely responsible for the activation of the ability of the ribosome for non-enzymatic translocation [4]. The action of other SH-reagents (iodoacetamide, *N*-ethylmaleimide) on the 30 S subparticle did not give such as effect and at the same time did not prevent the subsequent activating effect by PCMB [5]. Since S12 was found to be the only protein inaccessible for the alkylating SH-reagents among those accessible for PCMB, the conclusion was made that it is the modification of the protein S12 by PCMB that is responsible for activation of non-enzymatic translocation [5].

In the present communication it is shown that the ability for 'non-enzymatic' polyU-directed elongation and, therefore for non-enzymatic translocation in the ribosome is released (unblocked) by deletion of protein S12 from the 30 S subparticle as well as by its damage with PCMB. Conversely saturation of the 30 S subparticles with intact protein S12 prevents 'non-enzymatic' translation.

2. Materials and methods

Ribosomal 30 S and 50 S subparticles were prepared from *E. coli* MRE-600 by sucrose gradient zonal centrifugation in the presence of 0.5 M NH_4Cl with 1 mM $MgCl_2$ [6] using the B-XV rotor of the MSE SS-65 centrifuge.

The proteins were extracted from the 30 S subparticles with 67% acetic acid in the presence of 0.2 M $MgCl_2$ [7,8], and then fractionated on a phosphocellulose column [7]. For a thorough purification from protein S12, the fractions adjoining the S12 peak in the elution profile were rechromatographed on phosphocellulose and Sephadex G-100 columns. The composition and purity of the fractions were controlled by two-dimensional polyacrylamide gel electrophoresis [9]. The nomenclature of Wittmann et al. was used to designate the proteins [10].

The ribosomal 16 S RNA was obtained from the 30 S subparticle either by removing protein with 2 M LiCl-4 M urea [7,11], or by phenol deproteinization with 1% sodium dodecyl sulphate [12].

The reconstitution of the 30 S subparticles from 16 S RNA and ribosomal protein fractions was carried out in a buffer 30 mM Tris-HCl – 20 mM $MgCl_2$ – 330 mM KCl – 1 mM dithiothreitol (DTT), $pH_{20°}$ 7.4, during 1 hr at 40°C [13]; the molar protein:RNA ratio was about 2–3 for all the proteins, except protein S12 which was either not added (30 S[–S12]), or was added at a molar ratio of about 5–6 (30 S[+S12]).

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Treatment of 30 S subparticles (including 30 S [–S12] and 30 S [+S12]) with PCMB or DTT and the subsequent removal of excess reagent by Sephadex G-50 gel-filtration were done as described earlier [4].

In a special experiment 50 S subparticles were treated with antibodies against protein S12 (anti-S12 IgG) kindly presented by Dr G. Stöffler, Max-Planck-Institut für Molekulare Genetik, Berlin. The particles were incubated with the anti-S12 IgG at a molar ratio of 1:32 for 30 min in an ice-bath [14].

In experiments on 'non-enzymatic' translation, the reaction mixture was prepared in a buffer with 10 mM Tris–HCl – 100 mM KCl – 10 or 13 mM $MgCl_2$, $pH_{25°}$ 7.1; 20 μl of the mixture contained 5 μg of 30 S subparticles (either original 30 S or 30 S [–S12], or 30 S [+S12]), 10 μg of 50 S subparticles, 8–10 μg of polyU (K^+-salt, Calbiochem) and 30 μg of total tRNA containing 17–18 pmoles of [^{14}C]phenylalanyl-tRNA (the initial [^{14}C]phenylalanine was from Amersham, England, 513 mCi/mmole); the incubation was done at 25°C [4,5]. When mentioned, streptomycin sulphate (SM) was present in the incubation mixture at a 5×10^{-5} or 10^{-4} M concentration. The radioactivity of hot trichloroacetic acid insoluble [^{14}C]polyphenylalanine was determined as described earlier [1, 2]. The amount of the [^{14}C]polyphenylalanine, expressed in picomoles of phenylalanine residues, was plotted versus incubation time.

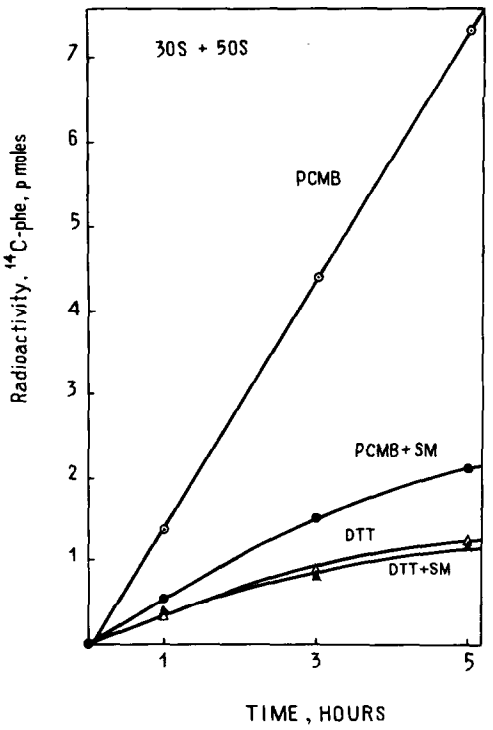

Fig. 1. [^{14}C]polyphenylalanine synthesis in the poly U-directed non-enzymatic system with the initial ribosomal subparticle preparation (30 S+50 S). ⊙ – particles treated with *para*-chloromercuribenzoate (PCMB); • – particles treated with PCMB, streptomycin (SM) added; △ – particles treated with dithiothreitol (DTT); ▲ – particles treated with DTT, SM added.

3. Results

3.1. *'Non-enzymatic' translation without treatment of ribosomes with PCMB*

It was observed that preparations of purified ribosomal subparticles usually contain a variable fraction (about 20–40%) displaying an ability for non-enzymatic translocation without PCMB pre-treatment (i.e., in the presence of DTT; see figs. in [4,5]). In contrast to PCMB-activated elongation this activity was found to be resistant against streptomycin (fig. 1).

There are three groups of data which can have a bearing on the above-mentioned observations. First, the resistance of ribosomes to streptomycin can be caused either by a mutational alteration of the ribosomal protein S12 or by its complete absence in the 30 S subparticle [15]. Second, there is evidence that the protein S12 in isolated 30 S subparticles is 'factional protein', i.e., it is absent in a fraction of the particles [16,17]. Third, activation of non-enzymatic translocation by PCMB can be due to the effect of the reagent simply on the protein S12 [5]. In such a case PCMB may destroy some function of protein S12 accounting for blocking of non-enzymatic translocation. As seen in fig. 1, this does not inactivate the other function of the protein which provides inhibition by streptomycin.

The data presented above lead to an assumption that the ability for non-enzymatic translocation without PCMB in a fraction of the ribosomal particles can be accounted for by the simple absence of protein S12. Thus, the ability for non-enzymatic translocation in the ribosome can be released (unblocked) both in the case of protein S12 damage by PCMB and *in the case of absence of protein S12 in the ribosome;* in the latter case the ribosomes will be streptomycin-resistant.

3.2. *'Non-enzymatic' translocation using the 30 S subparticles reconstituted in an excess of protein S12*

To test the above assumption we carried out experiments on the reconstitution of 30 S subparticles from 16 S RNA and a set of ribosomal proteins with a following assay of the reconstituted particles in a system of 'non-enzymatic' translocation. In a first series of experiments 30 S subparticles were reconstituted from a complete set of proteins *with an excess of protein S12* to ensure its presence in every particle. If the above-mentioned assumption (section 3.1) is correct, the non-enzymatic system with such a preparation must not exhibit polypeptide synthesis without PCMB treatment, and PCMB-activated synthesis must be inhibited by streptomycin. Data of one of such experiments are given in fig. 2. It is seen that saturation of the 30 S particles with protein S12 does lead to a strong decrease or practical absence of the ribosome fraction elongating without PCMB and resistant to streptomycin (the activity of this fraction does not exceed 10% of the full activity of particles with PCMB).

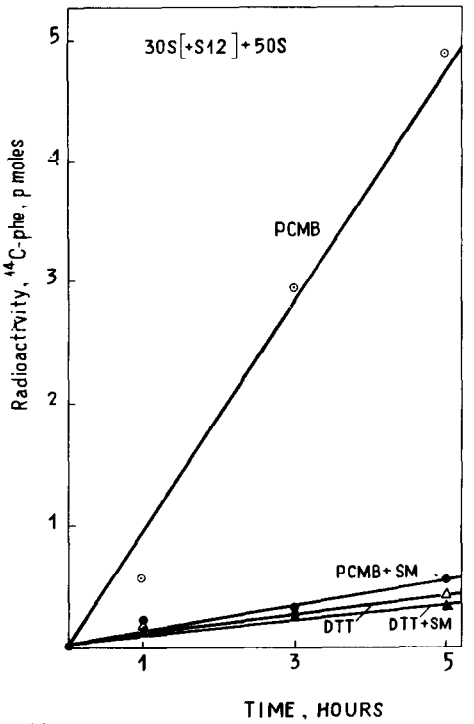

Fig. 2. [^{14}C]polyphenylalanine synthesis in the poly U-directed non-enzymatic system with 30 S subparticles reconstituted in an excess of protein S12, and with initial 50 S subparticles (30 S[+S12]+50 S).
⊙ – reconstituted 30 S subparticles pre-treated with *para*-chloromercuribenzoate (PCMB); • – reconstituted 30 S particles pre-treated with PCMB, streptomycin (SM) added; △ – reconstituted 30 S particles pre-treated with dithiothreitol (DTT); ▲ – reconstituted 30 S particles pre-treated with DTT, SM added.

3.3. *'Non-enzymatic translocation using 30 S subparticles reconstituted without protein S12*

In a second series of experiments 30 S subparticles were reconstituted from a set of proteins *excluding protein S12.* If the above-mentioned assumption (section 3.1.) and the interpretation of the previous experiment (section 3.2) are correct, the exclusion of protein S12 from the ribosome must give a system elongating non-enzymatically without PCMB (in the presence of DTT), not activated by PCMB and not inhibited by streptomycin. The data are presented in fig. 3. It is seen that ribosomes reconstituted without S12 indeed quite actively synthesize polyphenylalanine (about 60% of the full activity of the given sample of the particles)

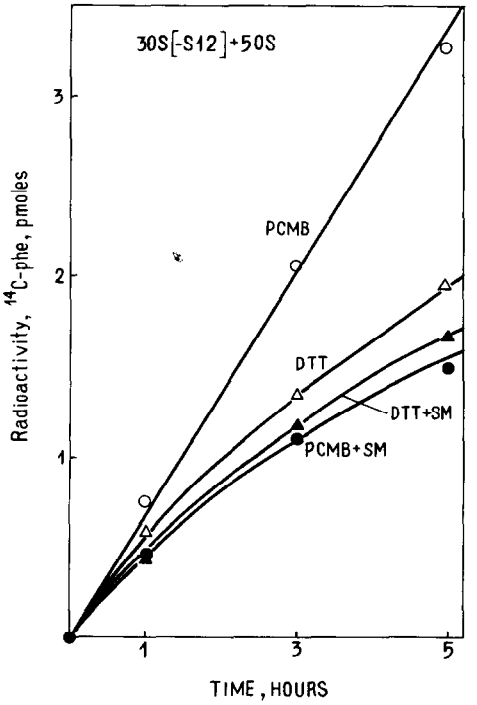

Fig. 3. [^{14}C]polyphenylalanine synthesis in the poly U-directed non-enzymatic system with 30 S subparticles reconstituted without protein S12, and with the initial 50 S subparticles (30 S[-S12]+50 S). Designations same as in fig. 2.

without PCMB-treatment and that this synthesis is streptomycin-resistant.

However, an essential stimulation of the synthesis by PCMB is observed in the experiment and this stimulation effect is removed by streptomycin (fig. 3). The latter can only indicate that despite exclusion of the protein S12 from the reconstituting protein mixture it seems to be present in some part of active ribosomes. There may be several reasons for this. The simplest one may be a contamination of original 30 S subparticles or their dimers in the 50 S subparticle preparations. That this reason does not play an essential role was shown in special control experiments where the peptide-synthesizing activity of 50 S subparticle preparations was tested in a complete ('enzymatic') polyU-directed cell-free system; according to this test the admixtures of functionally active 30 S subparticles in our 50 S subparticle preparations were not more than 2–3%. Sedimentation analysis also shows that contamination (if any) of the 30 S component in 50 S particle preparations does not exceed 5%. Another reason is the possibility of protein S12 admixtures in the fractions of other proteins. However, according to our electrophoretical analysis of the fractions, the admixture of protein S12 could not be more than 10%, while the observed effect of PCMB stimulation comprises about 40% of the full activity (fig. 3). Sucrose gradient centrifugation analysis of the 30 S subparticles reconstituted without S12 and treated with anti-S12 IgG according to Morrison et al. [18] also did not reveal observable aggregates or decrease of the 30 S peak. The third reason may be that the 50 S subparticles themselves can be a source of protein S12. Indeed, it was recently shown that a noticeable portion of pure isolated 50 S subparticles can contain protein S12 [18].

To exclude at least the last of the enumerated sources of protein S12, we used antibodies against protein S12 (anti-S12 IgG). Thus, a third series of experiments was carried out where 30 S subparticles were again reconstituted without protein S12 while the 50 S subparticle preparation was pre-incubated with anti-S12 IgG. The obtained 30 S subparticles without protein S12, pre-treated either with PCMB or DTT, were mixed with 50 S subparticles, still in the presence of anti-S12 IgG; then polyU and [^{14}C]-phenylalanyl-tRNA were added and 'non-enzymatic' translocation was followed. The results are given in fig. 4. It is seen that such a double exclusion of protein S12 from active ribosomes actually makes 'non-enzymatic' translocation practically independent of PCMB treatment and streptomycin.

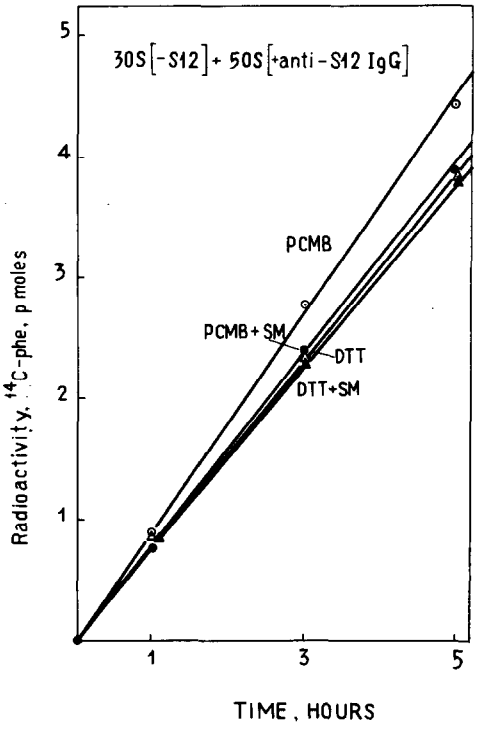

Fig. 4. [^{14}C]polyphenylalanine synthesis in the poly U-directed non-enzymatic system with 30 S subparticles reconstituted without protein S12, and with 50 S subparticles treated with anti-S12 IgG (30 S[-S12]+50 S [+anti-S12 IgG]). Designations same as in fig. 2.

4. Discussion

Non-enzymatic translocation in the absence of PCMB was first reported by Pestka [19,20], and then corroborated in our laboratory [21] (see also [22,23]). However we encountered poor reproducibility of this phenomenon in various preparations of pure ribosomes as well as with a low activity of such non-enzymatic systems. The use of PCMB [1] made the system of 'non-enzymatic' translocation much more active and of better reproducibility [23]. The data of a previous paper [5] and the present communication show that it is the protein S12 of the 30 S subparticle that has a

direct relation to non-enzymatic translocation: both its damage by PCMB and its complete deletion permits polyU-directed elongation in the absence of EF-T, EF-G and GTP. The data of this paper also indicate that the presence of intact protein S12 prevents such 'non-enzymatic' elongation. Thus, it may be thought that the ribosomes in Pestka's experiments [20,22] were strongly depleted of protein S12 during preparatory procedures and purification and hence displayed a relatively high activity in the non-enzymatic system, at the same time being little stimulated by *p*-chloromercuribenzenesulfonate (we cannot, however, exclude also the possibility of oxidation damage to protein S12 during the ribosome purification process without SH-compounds).

In either case the presented data indicate a previously unknown role of protein S12 in ribosome functioning. This protein prevents spontaneous (independent of EG-G and GTP) translocation in the ribosome. Consequently it must be in some way involved in the normal translocation mechanism.

It follows that the principle ability for translocation in the absence of intact protein S12, EF-G and GTP calls for a more attentive consideration of the role of the ribosome proper in the translocation mechanism. In any case the ability to carry out a translocation function and its mechanism seem to be inherent to the basic structure of the ribosome itself while the role of the factors and GTP may consist only in the energetic and kinetic promotion ('catalysis') of the function.

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