ELONGATION FACTOR G INTERACTS WITH BOTH RIBOSOMAL SUBPARTICLES

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1. Introduction

Translocation in the process of protein biosynthesis is known to be promoted by the interaction of the ribosome with GTP and the elongation factor G (EF-G) [1]. The knowledge of the ribosomal components forming the EF-G-binding site is one of the problems to be solved.

The present communication describes the preparation of a photoactivated arylazide derivative of EF-G in a highly radioactive form. Its use as an affinity reagent has shown that at the formation of a specific complex of the ribosome with EF-G the latter interacts (contacts) with both ribosomal subparticles.

2. Materials and methods

Ribosomes were obtained from *Escherichia coli* MRE 600 as in [2]. The ribosomal subparticles were separated by zonal centrifugation in the sucrose gradient in the presence of 0.5 M NH_4Cl and 1 mM $MgCl_2$ [3]. EF-G was obtained from *E. coli* MRE 600 by a combination of methods developed by [4,5] as described in [6]. *p*-Azidobenzaldehyde was synthesized according to [7]. The [^{14}C]GTP and NaB^3H_4 preparations (Amersham, England) had spec. act. 0.5 Ci/mmol and 6–8 Ci/mmol, respectively. NaB^3H_4 was dissolved in 0.3 M NaOH up to ~ 25 μmol/ml and stored under nitrogen in sealed ampoules at –60°C. The following buffers were used: A, 10 mM Hepes, pH 8.0, 5 mM β-mercaptoethanol; B, 0.6 M sodium borate, pH 9.0, 5 mM β-mercaptoethanol; C, 10 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 10 mM KCl; D, 10 mM Hepes, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, 500 mM NH_4Cl.

The photoactivated derivative of EF-G was synthesized in the dark at 0°C. *p*-Azidobenzaldehyde, 2 μmol in 0.2 ml buffer B, was mixed with EF-G, 25 nmol in 0.1 ml buffer A, and after 10 min NaB^3H_4 was added in portions (6 × 5 μl at 5 min interval). After the addition of the last portion of NaB^3H_4, the mixture was incubated for 30 min, then 60 μmol unlabeled borohydride dissolved directly before use in 60 μl buffer B were added and the mixture was incubated for 1 h. Upon completion of the reaction the mixture was dialyzed against buffer A (2 × 1 litres), and finally the product was purified by gel filtration on Sephadex G-50 in the same buffer. The [^{3}H]arylazido-EF-G preparation contained ~ 1–1.5 × 10^6 cpm/nmol EF-G, which corresponds to about 1 *p*-azidobenzaldehyde molecule/EF-G molecule.

The functional activity of the modified EF-G was tested in the dark by its ability to form a specific complex with the ribosome. The incubation mixture (0.3 ml) contained: 0.5 nmol 30 S and 50 S ribosomal subparticles each, 0.3 nmol [^{3}H]arylazido-EF-G, 1 nmol GTP and 0.7 μmol fusidic acid in buffer C. The mixture was incubated for 10 min at 25°C, and the complex was pelleted by centrifugation (Spinco L5-50, rotor Ti-50, 49 000 rev./min, 3 h, 4°C). The extent of EF-G binding was determined as a % ratio of the pellet radioactivity versus the total radioactivity of the sample. In the control experiment the same mixture and procedure were used, but the native EF-G and [^{14}C]GTP were taken instead of the modified EF-G and GTP, respectively. The extent of the native EF-G binding with the ribosome was determined as a molar ratio of the [^{14}C]GTP in the pellet versus the total amount of EF-G in the sample (this calculation is based on the known data that the

ribosome·EF-G·GTP complex contains equimolar amounts of EF-G and the nucleotide [8].

The mixture was irradiated with mercury lamp SVD-120A (USSR) equipped with a filter to cut-off radiation below 300 nm. Irradiation time was 3 min at 4°C (complete photolysis of the arylazide group).

The distribution of the radioactive label between the ribosomal subparticles was analyzed after dialysis of the irradiated complex (1 ml) in the cold against the dissociating buffer D and the separation of the components by sucrose gradient centrifugation (5–20% sucrose, 30 ml, in buffer D, Spinco L5-50, Sw-25,1 rotor, 24 000 rev./min, 12 h, 4°C.

3. Results and discussion

The choice of *p*-azidobenzaldehyde as a bifunctional reagent for obtaining a chemically active EF-G derivative and the use of the latter for affinity labeling of the ribosomal EF-G-binding site is determined by the following reasons:

1. As known, aldehyde treatment of proteins in the presence of NaB^3H_4 (reductive alkylation) results in a highly specific modification of the amino groups without a change in their charge, which permits, in most cases, to preserve the biological activity of protein and to introduce the radioactive label into the modified point (see, e.g. [9–11]).

2. Arylazides have two features important for us:
(i) A controlled chemical reactivity (stability in the dark and mild activation at irradiation with a wave-length above 300 nm).
(ii) A high activity of the light-generated nitrene radical which can attack any sterically close bond, including the C-H [12].
The first feature (i) of arylazides excludes possible inter- or intramolecular cross-linking in the course of treatment of the EF-G with the reagent and during isolation and functional testing of the EF-G derivative (if these procedures are carried out in the dark) and to 'switch on' the labeling of the ribosome with the arylazido-EF-G at the proper moment (after the formation of the EF-G complex with the ribosome).
The second feature (ii) of arylazides ensures labeling of the ribosome regardless of the nature of the components exposed near its EF-G-binding site.

The functional activity of the [^{3}H]arylazido-EF-G obtained was tested by its ability to form a specific complex with the ribosome. As a criterium of its specificity we chose the effect of GTP, which is required for EF-G binding to the ribosome, and also that of fusidic acid which stabilizes the complex and thus increases its yield [13,14]. For comparison, we analyzed the extent of native EF-G binding. The results are given in table 1. It is seen that the modification of EF-G affects neither the extent of its binding with ribosome nor the specificity of this complex formation.

Figure 1A (●—●) shows the labeling of the ribosomal subparticles at irradiation of the

Table 1
Analysis of the ability of [^{3}H]arylazido-EF-G to form a specific complex with the ribosome

EF-G preparation	Ribosome	GTP[a]	Fusidic acid	Extent of binding to the ribosome (%)
[^{3}H]Arylazido-	+	+	+	72
	+	–	–	5.1
	–	–	–	0.3
Native	+	+	+	71
	+	+	–	9.1

[a] [^{12}C]GTP and [^{14}C]GTP were used in experiments with the derivative of EF-G and native EF-G, respectively

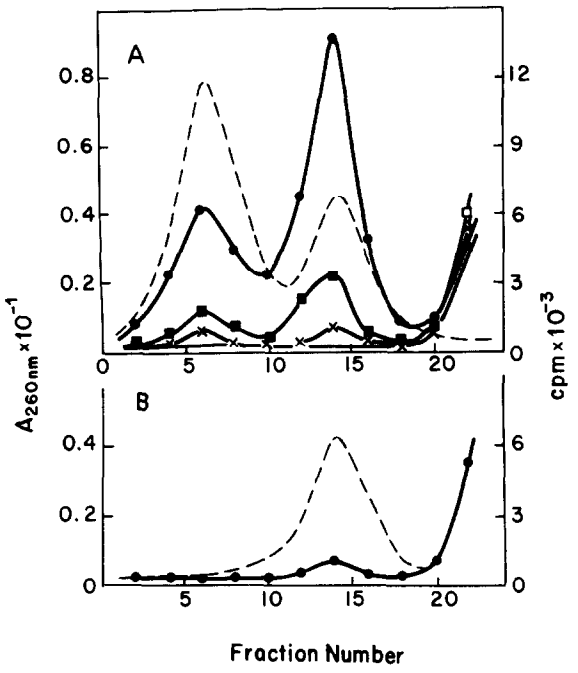

Fig.1(A) Photolabeling of ribsomal subparticles with [^{3}H]-arylazido-EF-G: (- - -) A_{260}; (●—●) mixture of 30 S + 50 S + [^{3}H]arylazido-EF-G + GTP + fusidic acid; (■—■) the same as (●—●) but without the nucleotide and fusidic acid; (X—X) the same as (■—■) but + thiostrepton (1×10^{-5} M); (——) the same as (●—●) but without irradiation. (B) Photolabeling of the ribosomal 30 S subparticle with [^{3}H]arylazido-EF-G in the presence of GTP and fusidic acid; (- - -) A_{260}; (●—●) radioactivity.

ribosome·GTP·[^{3}H]arylazido-EF-G complex in the presence of fusidic acid. It is seen that ribosome labeling (its effectivity was about 10%) is a photoactivated reaction (cf. (●—●) and (——)). The main result of the experiment is the appearance of a covalently bound label in both ribosomal subparticles as a result of irradiation. This labeling is highly specific as it sharply decreases in the case of irradiation of the ribosome with EF-G derivative without GTP (■—■) and is completely blocked in the presence of antibiotic thiostrepton (X—X) which, as known [15,16], affects the ribosome inhibiting its interaction with EF-G. Thus, the photoactivated labeling of both ribosomal subparticles with [^{3}H]arylazido-EF-G is a true affinity reaction.

A comparison of the data in fig.1A (●—●) and 1B shows that the label in the 30 S subparticle appears only at irradiation of the EF-G derivative with the whole ribosome, while irradiation of the [^{3}H]arylazido-EF-G with the isolated 30 S subparticle does not result in essential labeling of the latter. In other words, the specific interaction (contact) of EF-G with the 30 S subparticle is detected only after association of this particle with the 50 S subparticle. It can be noted that the presence of a direct interaction of EF-G with the 30 S subparticle was suggested [17] on the basis of *p*-hydroxymercuribenzoate transition from SH-group of EF-G to the 30 S subparticle. But this transition as seen from experimental data in [17] is evidently not caused by the formation of EF-G complex with the ribosome.

It is noteworthy that irradiation of [^{3}H]arylazido-EF-G with the ribosome in the absence of GTP also leads to some affinity labeling of both subparticles (fig.1A (■—■); cf. (X—X)), though the effectivity of the reaction is essentially lower as compared with that for the complex with GTP. This fact confirms the existence of a specific affinity of EF-G for the ribosome even without GTP as observed [18]. The similarity of the profiles of the label distribution between the ribosomal subparticles for ribosome·[^{3}H]arylazido-EF-G complexes in the presence of GTP and without it (fig.1A (●—●), (■—■)) suggests that the EF-G molecule settles on the ribosome in both complexes in a same manner.

Thus, the use of the photoactivated EF-G derivative reveals two features of the EF-G binding to the ribosome:

1. The interaction of EF-G with both ribosomal subparticles, if they are in the associated state.
2. The presence of some specific affinity of EF-G for the ribosome without GTP.

These results seem to be important for understanding the nature of EF-G functioning and the mechanism of translocation as a whole. In particular it is known that the 50 S subparticle can be sufficient for EF-G binding [19–21]. The isolated 30 S subparticle is not active in this process, but its addition to the 50 S subparticle stimulates significantly the binding of EF-G [22–28]. According to our data, this effect of the 30 S subparticle may be explained by the appearance of a specific interaction of EF-G with the 30 S subparticle associated with the 50 S subparticle,

thus leading to an additional stabilization of the EF-G complex with the ribosome.

Some authors have indicated that GTP (or its unhydrolyzable analog) is a specific allosteric effector inducing the EF-G joining to the ribosome for translocation of peptidyl-tRNA [29–31]. But, according to the data in [18] and our results, EF-G has a specific affinity for the ribosome even without GTP. Therefore the possibility of translocation by EF-G in the absence of nucleotide could be predicted. However, in this case the effective translocation evidently needs a significant molar excess of EF-G because of the lability of EF-G·ribosome complex without GTP.

In conclusion it should be noted that the result obtained agrees with Spirin's hypothesis proposed 10 years ago [32] that both subparticles must be involved in the ribosomal translocation mechanism.

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