STUDIES ON TRANSLOCATION V: FUSIDIC ACID STABILIZATION OF A EUKARYOTIC RIBOSOME-TRANSLOCATION FACTOR–GDP COMPLEX*

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1. Introduction

Translocation, the movement of the ribosome along mRNA, is driven by the hydrolysis of GTP in both prokaryotic [1, 2] and eukaryotic [3–5] systems and requires the participation of a soluble protein, factors G and T2, respectively. The broad outlines of this process in these two systems appear similar. However, significant differences must exist because the two types of ribosomes are structurally distinct and none of the components are active in the heterologous system [6, 7]. But despite these differences, the steroidal antibiotic, fusidic acid, inhibits protein synthesis in both prokaryotic [8–10] and eukaryotic [11, 12] cell-free systems by interfering with the translocation step.

The interaction of the prokaryotic translocation factor G with ribosome has been shown to involve the transient formation of a ternary complex involving the ribosome, G factor, and GDP [13]. Despite the fact that fusidic acid inhibits GTP hydrolysis by this system, the antibiotic enhances the yield of this complex under conditions where bound GDP has arisen from GTP [14]. This finding suggested to us that the antibiotic may inhibit repetition of this process by preventing the dissociation of the product-containing complex. Subsequent experiments have supported this view [15, 16].

The present experiments were designed to examine this interaction and the effect of fusidic acid in a eukaryotic system, one derived from rabbit reticulocytes. We sought to determine if a complex is formed analogous to that exhibited by the *E. coli* components and if fusidic acid enhances is stability. We have observed this complex and while it is intrinsically more stable than its *E. coli* counterpart, its stability is further increased by fusidic acid.

2. Results

Factor T2 is known to bind to ribosomes in a guanine nucleotide-dependent reaction [17–19], although no direct evidence has been presented to define the participation of the nucleotide in this interaction. When mixtures of *E. coli* G factor and ribosomes and ^{3}H- and γ-^{32}P-GTP are passed rapidly through columns of Bio-gel A-1.5 m†, ribosome-bound GDP (^{3}H but not ^{32}P) appears in the column void volume [14]. A comparable experiment exploying the reticulocyte counterparts is shown in fig. 1A. A large well-defined peak of ^{3}H is apparent at the

* The previous paper in this series is [16].

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† Because of the high exclusion limit of this gel (ca. 1.5×10^{6} daltons for globular poteins) only ribosomes and low molecular weight components bound to ribosomes are excluded.

column void volume. This peak is well resolved from nucleotide which is included by the column and the latter, but not the former, peak contains ^{32}P.

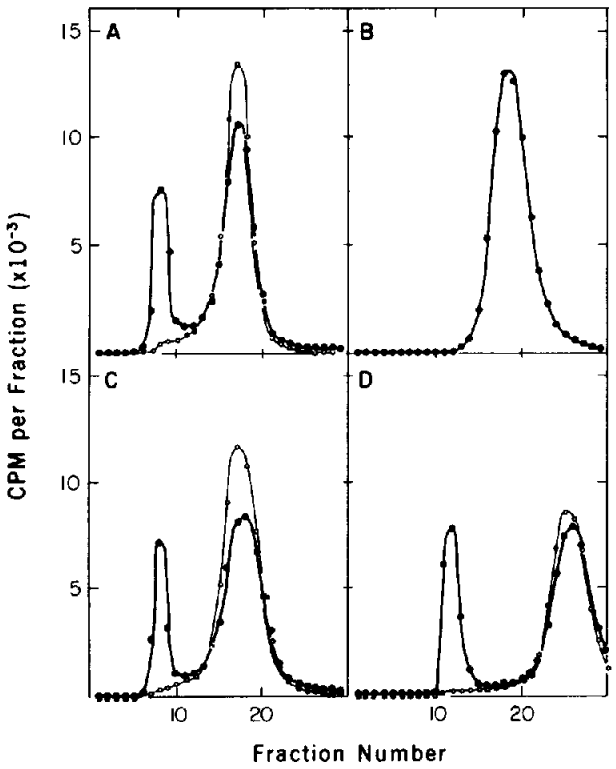

Fig. 1. Detection of ribosome-bound nucleotide by gel filtration. Reticulocyte ribosomes (200 μg), factor T2 (37 μg) and GTP (53 pmoles; ca., 65,000 cpm each ^{3}H- and γ-^{32}P-GTP) were reacted for 5 min at 0° in a final volume of 50 μl buffer A (10 mM tris-Cl pH 7.4, 10 mM NH_4Cl, 10 mM $MgAc_2$ and 1.0 mM dithiothreitol). Following incubation, the reaction mixture was placed on a 0.65 × 6.5 cm column of Bio-Gel A-1.5 m and eluted with buffer A at a flow rate of 0.1 ml/min. Ten-drop fractions (ca. 0.1 ml) were collected and the radioactivity in the entire fraction was determined in a liquid scintillation counter. Ribosomes and factor were prepared as previously described [3]. Other methods and the sources of materials are reported in detail elsewhere [14, 20].

A) complete reaction,

B) complete reaction minus T2,

C) same as A plus 3 mM fusidic acid (kindly supplied by Miss Barbara Stearns of Squibb) in reaction mixture and 0.1 mM in elution buffer,

D) same as A except that 3 mM fusidic acid was present throughout.

●—● ^{3}H, ○—○ ^{32}P.

Control experiments of the type shown in fig. 1B indicate, as is the case with the *E. coli* components, that the binding of tritium-labeled nucleotide (GDP) is completely dependent on both ribosomes and T2. No radioactivity is excluded from these Bio-gel columns if either ribosomes or factor are omitted from the reaction mixture. The experiments shown in figs. 1C and 1D are identical to that in A except that 3 mM fusidic acid was present in the reaction mixture. This high concentration of antibiotic is more than sufficient completely to suppress protein synthesis by the reticulocyte system [11]. In fig. 1C, 0.01 mM fusidic acid was also present in the elution buffer. The elution pattern is essentially identical to that obtained without the antibiotic. When fusidic acid was present at a high concentration in both the reaction mixture and the elution buffer (fig. 1D), the pattern is shifted to the right, presumably as a result of decreased drop size caused by the presence of fusidic acid, but the yield of bound nucleotide is only slightly increased over that observed in the total absence of the antibiotic. Under these conditions, the yield of bound nucleotide with the *E. coli* system is enhanced approximately 5-fold by fusidic acid [14].

The method of millipore filtration was also used to assess this nucleotide interaction and the effect of fusidic acid. Factor T2 has been reported to bind GTP [21], but by contrast, no interaction between G factor and the guanine nucleotide has been demonstrates. Experiment 1 (table 1) shows that this T2 preperation causes the retention of GTP (both ^{3}H- and γ-^{32}P) by millipore filters. GTP is also retained and fusidic acid has no effect on the binding of either nucleotide (exp. 2). The addition of reticulocyte ribosomes to this system causes an increase in the retention of ^{3}H from either GTP or GDP but does not alter the binding of ^{32}P (exp. 3)*. Exp. 4 (table 1) demonstrates that the

* This latter result casts doubt on the significance of the binding of nucleotide by T2 preparations. If this binding is a true intermediate to the interaction with the ribosome, then the addition of ribosomes should reduce the level of ^{32}P binding because this isotope is not retained on the ribosome (fig. 1). However, two different T2 preparations of comparable specific activity exhibited approximately equivalent GTP binding. In any event, binding to protein does not appear to involve significant GTP hydrolysis, nor is this binding altered by fusidic acid.

Table 1
Detection of bound nucleotide by Millipore filtration.

Experiment	Nucleotide retained (pmoles)		
	^{3}H-GTP	γ-^{32}P-GTP	^{3}H-GDP
1) T2 alone	1.9	2.8	1.0
2) T2 + fusidic acid	2.0	3.0	1.1
3) T2 + ret. ribo	7.7	2.1	6.1
4) T2 + ret. ribo + fusidic acid	9.6	2.5	8.5
5) G + *coli* ribo + fusidic acid	9.5	0.1	10.1
6) G + *coli* ribo	3.0	0.1	3.6
7) T2 + *coli* ribo	1.7	2.2	–
8) G + ret. ribo	0.6	0.2	–

Incubation was conducted as described in fig. 1.Where indicated, 18.5 μg of factor T2 or 7.4 μg factor G was present. Reticulocyte (ret.) and *E. coli* ribosomes (ribo) were present at 100 μg and 125 μg per reaction, respectively. The fusidic acid concentration was 3 mM. When GDP binding was measured, each reaction contained 72,000 cpm ^{3}H-GDP (58 pmoles). Following incubation, the reaction mixture was diluted with buffer A (fig. 1) and filtered and washed on Millipore filters as described elsewhere [20]. *E. coli* ribosomes and G factor were prepared as previously described [20] except that the factor was further purified by hydroxylapatite chromatography and filtration on Sephadex G-100.

presence of fusidic acid leads to a small but reproducible increase in GDP retention by the reticulocyte ribosome–T2 combination. This can be contrasted to the significant increase in binding produced by the antibiotic when the components are derived from *E. coli* (table 1, exps. 5 and 6). Exps. 7 and 8 explore the species specificity of nucleotide binding. *E. coli* ribosomes do not increase the binding of nucleotide by T2 (exp. 7). No nucleotide is retained by the G factor–reticulocyte ribosome combination (exp. 8) indicating not only that the combination is inactive in this regard but that neither component causes nucleotide retention.

By the criteria of both gel and millipore filtration, GDP binds much more tightly to reticulocyte than *E. coli* ribosomes in the reactions brought about by their respective translocation factors; so tightly, in fact, that neither of these methods allows a direct assessment of possible stabilization of the reticulocyte complex by fusidic acid as is the case with the *E. coli* ribosome–G factor–GDP complex. If such stabilization is occurring, however, then fusidic acid should prevent the chase of previously bound labeled nucleotide by an excess of unlabeled GTP. An experiment designed to test this possibility is shown in fig. 2. Labeled GTP was incubated with T2 and the T2–ribosome combination in the presence and absence of fusidic acid. After binding, an excess of unlabeled GTP was added and the rate of labeled complex disappearance was followed at 30° in the three cases. Radioactive nucleotide (GTP) bound to T2 alone disappears rapidly under these chase conditions. The addition of ribosomes leads to an increase in nucleotide (GDP) binding, but again the labeled nucleotide is completely chased from the complex, although somewhat more slowly than with T2 alone. In the presence of T2, ribosomes and fusidic acid, there is a rapid loss of labeled complex corresponding approximately to that amount of nucleotide bound by T2 alone. Beyond this amount, however, bound ^{3}H-nucleotide is displaced very slowly by unlabeled GTP.

The stabilization of bound GDP by fusidic acid has been further demonstrated by conducting gel filtration at room temperature, in the presence and absence of the antibiotic, of a complex formed with limiting ^{3}H-GTP. Under these conditions and at a flow rate one-half that employed in fig. 1, the presence of fusidic acid produced a three-fold increase in bound nucleotide.

3. Discussion

While the mammalian translocation factor is known

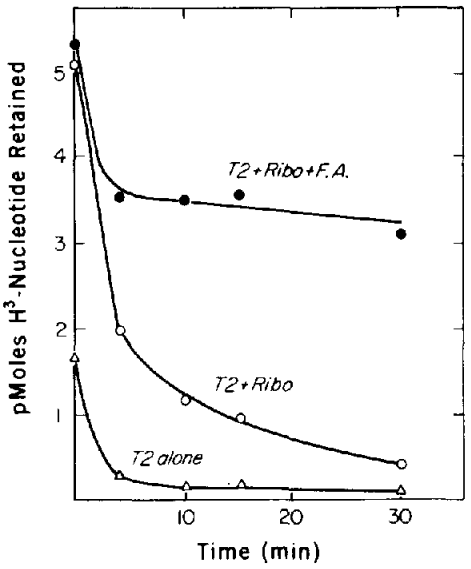

Fig. 2. The rate of chase by unlabeled GTP of bound, labeled nucleotide. Incubation with ^{3}H-GTP (280 pmoles) was conducted as described in fig. 1 except that the reaction was scaled up to 300 µl. Following this incubation, 1.0 mM unlabeled GTP was added and the reaction temperature was raised to 30°. At the indicated times, 50 µl aliquots were removed and filtered on Millipore filters as described in table 1.

to bind to ribosomes in a reaction dependent on GTP, the present experiments provide, to our knowledge, the first direct demonstration of the nucleotide participation in this association and reveal a significant parallel between the eukaryotic and prokaryotic systems. This interaction in both cases appears to lead to the formation of a relatively stable ribosome-translocation factor–GDP complex with the release of P_i. The major difference between the two systems is in the intrinsic stability of the eukaryotic complex, although both complexes are sufficiently stable to be isolated by gel filtration. The steroidal antibiotic, fusidic acid, appears to operate in both cases further to enhance complex stability and therefore indirectly to inhibit this interaction by preventing its repetition. While neither the translocation factors nor the ribosomes are active in the heterologous system, in either translocation or complex formation, the fact that fusidic acid inhibits both systems by a common mechanism suggests that both possess common functional properties.

Acknowledgements

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