FORMATION OF THE RIBOSOME-G FACTOR-GDP COMPLEX IN THE PRESENCE OF FUSIDIC ACID

James W. Bodley, Franklin J. Zieve*,

Lillian Lin and Sandra T. Zieve

Department of Biochemistry, University of Minnesota
Medical School, and Radioisotope Service,
Veterans Administration Hospital,
Minneapolis, Minnesota 55455

Received August 22, 1969

SUMMARY: When ribosomes are incubated with G factor and GTP, a ribosome-G factor-GDP complex is formed. Fusidic acid does not inhibit the formation of this complex; in fact, more complex is isolated in the presence of the antibiotic (10^{-3} M) than in its absence. No complex containing GTP is detectable in the presence or absence of the antibiotic.

Translation of genetic information requires the stepwise movement of the ribosome along mRNA. This movement of the ribosome involves the translocation of the peptidyl-tRNA-mRNA complex from the aminoacyl site to the peptidyl site on the ribosome, where the peptidyl-tRNA can then participate in the formation of a new peptide bond (1-3). The translocation process derives its energy from the hydrolysis of GTP to GDP and P_i and specifically requires the soluble transfer factor G (2-5). In addition to catalyzing translocation, G factor can also hydrolyze GTP in the presence of ribosomes in a reaction uncoupled from protein synthesis (2,6,7). The mechanism of both of these reactions remains obscure.

The steroidal antibiotic, fusidic acid, has recently been shown to inhibit protein synthesis by preventing translocation (4,8). Fusidic acid also inhibits the uncoupled GTPase activity of G factor (8,9). Recent

---

*Medical Scientist Fellow of the Life Insurance Medical Research Fund.

evidence indicates that this latter reaction is accompanied by the formation of a ternary complex involving ribosomes, G factor and GDP, perhaps as an intermediate in the reaction (10,11). The present experiments were designed to determine how fusidic acid influences the formation and breakdown of this complex.

MATERIALS AND METHODS

Fusidic acid was a generous gift from Squibb. E. coli B was obtained from Grain Processing Corp. H^3-GTP (specific activity 1.4 C/mmole) and γ-P^{32}-GTP (initial specific activity 9.4 C/mmole) were from Schwartz Bioresearch and from International Chemical and Nuclear Corp., respectively. The sources of other materials were as previously described (12).

Ribosomes were washed seven times with 0.01 M Tris-Cl, pH 7.4, containing in the first wash 1.0 M NH_4Cl and 0.0001 M $MgAc_2$, and in the subsequent washes 0.5 M NH_4Cl and 0.01 M $MgAc_2$. G factor was purified by DEAE-Sephadex chromatography according to the procedure of Lucas-Lenard and Lipmann (13). The enzyme was assayed by complementation with T factor in the polymerization of Phe-tRNA. G factor alone had no detectable polymerizing activity. The GTPase activity of the enzyme was assayed by the method of Nishizuka and Lipmann (6). At a substrate concentration of 3×10^{-3} M, it hydrolyzed 4.3 µmoles GTP/mg protein in 10 min. at 37°. This hydrolysis was 92% dependent upon ribosomes and, in the presence of ribosomes, was inhibited 92% by 10^{-3} M fusidic acid.

The ribosome-G factor-GDP complexes were formed and quantitated essentially as described by Brot, et al. (10). The reaction mixture (50 µl), unless otherwise specified, contained standard buffer (0.01 M Tris-Cl, pH 7.4, 0.01 M $MgAc_2$, 0.01 M NH_4Cl, 0.001 M dithiothreitol), 826 pmoles GTP (10^6 cpm each of H^3- and γ-P^{32}-GTP), 37.5 µg G factor and 140 pmoles ribosomes. After incubation for 5 min. at 0°, the sample was placed on a 6.5 x 0.65 cm column of Sephadex G-25 (medium) equilibrated with standard buffer. The column was eluted with standard buffer at a flow rate of approximately 0.1 ml/min., and five drop fractions (ca. 50 µl) were collected directly from the tip of a

23 ga. needle. A 10 µl aliquot of each fraction was taken for the determination of H^3 and P^{32} in a liquid scintillation counter. Ribosomes were quantitated by their A_{260}, assuming an $E^{1\%}_{260}$ of 145 and a M.W. of 2.7×10^6.

For the chromatographic identification of the bound nucleotide, the reaction mixture was scaled up two fold and passed through a column of Bio-gel A 1.5m. 20 µl of 100% TCA was added to the pooled breakthrough peak (~200 µl), the mixture was centrifuged, and the supernatant was extracted with ether to remove TCA. 5. µl, containing 15 pmoles radioactive nucleotide, was spotted on a Baker Flex PEI cellulose thin layer sheet along with 10 nmoles each of marker GTP, GDP and GMP, and developed in a 1.5 x 15 cm channel with 2 M acetic acid, 1 M LiCl (14). The sheets were washed with absolute methanol before and after chromatography, and after drying, the U.V. absorbing spots were cut out and counted in a liquid scintillation counter.

RESULTS AND DISCUSSION

In agreement with the observations of Brot, et al. (10), the simultaneous presence of ribosomes and G factor caused GDP, but not GTP, to be excluded from Sephadex G-25 (Fig. 1A). As shown in Table I, exp. 1, 10-15% of the ribosomes contained bound GDP. The amount of γ-P^{32}-GTP in the void volume was not detectably greater than that which occurred in the presence of G factor alone.

Fusidic acid could have several possible effects on the formation of this ternary complex. If the antibiotic inhibited GTPase by preventing complex formation, then it should prevent the exclusion of H^3-GDP from Sephadex G-25. If, on the other hand, it blocked the hydrolytic reaction per se, it might stabilize a complex containing both H^3- and γ-P^{32}-GTP.

Surprisingly, complexes formed in the presence of 10^{-3} M fusidic acid were indistinguishable from those formed in its absence (Fig. 1B and Table I, exp. 2). When fusidic acid was present on the column as well as in the reaction mixture, however (Fig. 1C), there was a marked increase in excluded H^3 with no increase in excluded P^{32}. Under these conditions approximately 1 mole of H^3-GDP was bound per 2 moles of ribosomes (Table I, exp. 3). Complex for-

TABLE I. Stoichiometry of nucleotide binding.

	Experiment	moles H^3-GDP / mole ribosomes	moles γ-P^{32}-GTP / mole ribosomes
1.	Complete reaction	0.13	0.01
2.	Fusidic acid in reaction	0.19	0.02
3.	Fusidic acid in reaction and on column	0.54	0.02
4.	Same as #3 except minus G factor	0.01	--
5.	Same as #3 except with 35 pmoles ribosomes	0.26	--
6.	Same as #3 except with 7.5 µg G factor	0.08	--
7.	Same as #3 except with 72 pmoles H^3-GTP	0.31	--
8.	Same as #3 except chromatographed on Bio-gel A 1.5m	0.47	--

Complexes were formed and chromatographed as described in MATERIALS AND METHODS with the omissions indicated. The columns employed in experiments 3-8 were equilibrated and eluted with buffer containing 10^{-3} M fusidic acid. In order to obviate variations due to fluctuations in fraction size, the amounts of radioactive nucleotide and ribosomes occurring in 10 µl aliquots of the fractions comprising the breakthrough peak were totaled. The amount of H^3-GDP and γ-P^{32}-GTP (0.65 and 0.59 pmoles, respectively) appearing in this region in the absence of ribosomes was subtracted from all of the data.

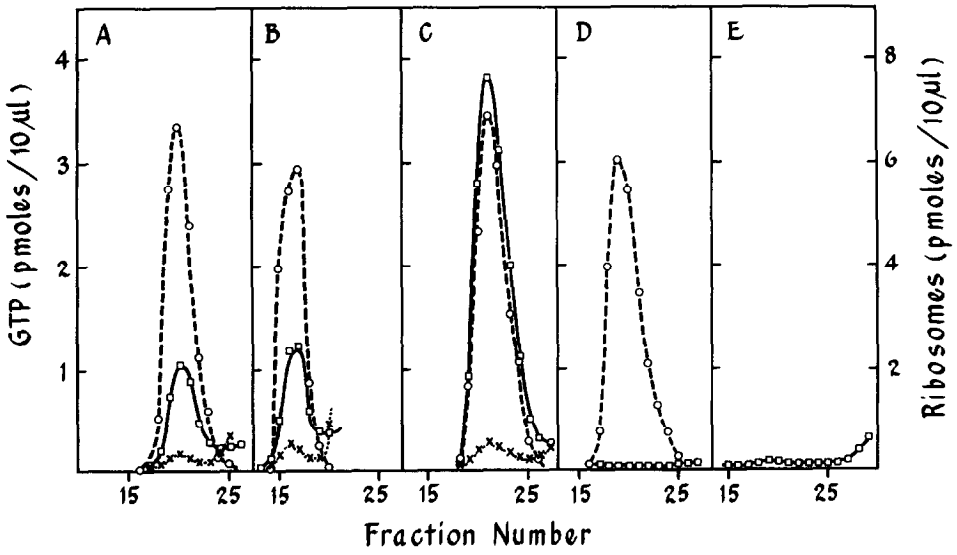

Figure 1. Sephadex G-25 gel filtration of ribosome-G factor-GDP complexes. Unless otherwise specified the complexes were formed and filtered as described in MATERIALS AND METHODS. A. Complete reaction. B. Complete reaction containing 10^{-3} M fusidic acid. C. Complete reaction containing 10^{-3} M fusidic acid filtered on a column equilibrated with buffer containing 10^{-3} M fusidic acid. D. Same as C. except G factor was omitted. E. Same as C. except ribosomes were omitted. □——□, pmoles H^3-GTP; x...x, pmoles γ-P^{32}-GTP; o---o, pmoles ribosomes.

mation in the presence of fusidic acid required both ribosomes and G factor, as shown in Fig. 1D and E.

The possibility that these complexes arise from the stoichiometric combination of G factor, ribosomes and GDP was supported by the observation (Table I, exp. 5 and 6) that reducing the concentration of either G factor or ribosomes caused an approximately proportional decrease in bound H^3-GDP. When the GTP concentration was reduced from 1.65×10^{-5} M to 1.4×10^{-6} M, binding was reduced only slightly, and the amount of bound GDP corresponded to about 60% of the total GTP incubated. The K_m for GTP in this system (determined, of course, in the absence of fusidic acid) is 2×10^{-6} M (2).

While all three components are required for the formation of the GDP-containing complex in the presence of fusidic acid, it is possible that all three might not be retained when the complex is isolated. G factor is reported to have a molecular weight of approximately 80,000 (7,11), and therefore should not appear in the void volume of a column of Bio-gel A 1.5m, which has an exclusion limit of ca. 1.5×10^6. The complex involving H^3-GDP was excluded from such a column (Table I, exp. 8). Similarly, G factor appeared in the breakthrough peak when incubated with GTP, ribosomes and fusidic acid, but not when incubated in the absence of ribosomes (Fig. 2). Because of the technical problems involved in assaying G factor in the presence of fusidic acid, we have not determined if the enzyme is excluded from columns washed with the antibiotic

The involvement of ribosomes in the complex was further demonstrated by the retention of H^3-GDP on Millipore filters washed with buffer containing fusidic acid. In one experiment complexes containing 1.9 pmoles H^3-GDP were filtered in the presence of 10^{-3} M fusidic acid; 1.3 pmoles were retained on the filter. Retention of complexes formed and filtered in the absence of fusidic acid was very low. No radioactivity was retained when the filters were washed with 5% TCA.

Direct demonstration of the identity of the nucleotide bound in the presence of fusidic acid is provided in Table II. The bound radioactivity, after

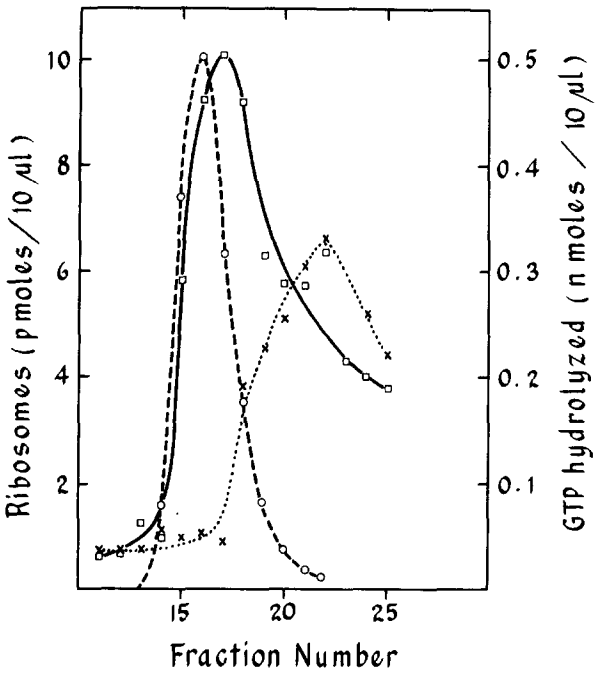

Figure 2. Binding of G factor to ribosomes in the presence of fusidic acid. Reactions containing 10^{-3} M fusidic acid were incubated and run on a Bio-gel A 1.5m column as described in MATERIALS AND METHODS. Aliquots were assayed for GTPase activity by the method of Nishizuka and Lipmann (6). The reaction mixture (50 µl) contained, in addition to buffer and salts, 76 µg ribosomes, 2 x 10^{-5} M γ-P^{32}-GTP (50,000 cpm) and 10 µl of the column fraction. Incubation was for 10 min. at 30°. o---o, pmoles ribosomes; □—□, GTPase activity (ribosomes present during complex formation); x...x, GTPase activity (ribosomes absent during complex formation).

TABLE II. Chromatographic identification of the bound nucleotide.

Marker	R_f	Percent radioactivity
GTP	0.14	1.2
GDP	0.47	97.3
GMP	0.73	<0.5

H^3 labeled complexes were isolated, deproteinized and co-chromatographed with marker nucleotides as described in MATERIALS AND METHODS. R_f's were calculated from the position of the U.V. absorbing marker spots. To obviate quench correction, the radioactivity is expressed as percent of a sample which was similarly spotted but prevented from migrating by a horizontal groove in the sheet.

---

isolation and deproteinization of the complexes, co-chromatographed with marker GDP.

Clearly, fusidic acid does not prevent the formation of the ternary complex involving ribosomes, G factor and GDP; in fact it promotes the isolation of the complex. While other interpretations are possible, the present data are consistent with the view that fusidic acid inhibits GTP hydrolysis by preventing the dissociation of the GDP containing complex rather than by inhibiting the hydrolytic reaction per se. Experiments are in progress to test this possibility and to further delineate the nature of the complex and its relationship to the translocation process.

ACKNOWLEDGMENTS: This work was supported by grants from the American Cancer Society (P-455) and from the Graduate School of the University of Minnesota.

REFERENCES

1. Watson, J.D., Bull. Soc. Chim. Biol., 46, 1399 (1964).
2. Nishizuka, Y., and Lipmann, F., Arch. Biochem. Biophys., 116, 344 (1966).
3. Erbe, R.W., Nau, M.M., and Leder, P., J. Mol. Biol., 38, 441 (1969).
4. Pestka, S., Proc. Nat. Acad. Sci. U.S.A., 61, 726 (1968).
5. Haenni, A.-L., and Lucas-Lenard, J., Proc. Nat. Acad. Sci. U.S.A., 61, 1363 (1968).
6. Nishizuka, Y., and Lipmann, F., Proc. Nat. Acad. Sci. U.S.A., 55, 212 (1966).
7. Leder, P., Skogerson, L.E., and Nau, M.M., Proc. Nat. Acad. Sci. U.S.A., 62, 454 (1969).
8. Tanaka, N., Kinoshita, T., and Masukawa, H., Biochem. Biophys. Res. Communs., 30, 278 (1968).
9. Kinoshita, T., Kawano, G., and Tanaka, N., Biochem. Biophys. Res. Communs., 33, 769 (1968).
10. Brot, N., Spears, C., and Weissbach, H., Biochem. Biophys. Res. Communs., 34, 843 (1969).
11. Parmeggiani, A., and Gottschalk, E.M., Biochem. Biophys. Res. Communs., 35, 861 (1969).
12. Bodley, J.W., and Zieve, F.J., Biochem. Biophys. Res. Communs., 36, 463 (1969).
13. Lucas-Lenard, J., and Lipmann, F., Proc. Nat. Acad. Sci. U.S.A., 55, 1562 (1966).
14. Randerath, K., and Randerath, E., J. Chromatog., 16, 111 (1964).