FORMATION OF FUSIDIC ACID-G FACTOR-GDP-RIBOSOME COMPLEX AND THE RELATIONSHIP TO THE INHIBITION OF GTP HYDROLYSIS

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Fusidic acid increases the binding of GTP with ribosomes and G factor. GTP is hydrolyzed to GDP in the complex with or without the antibiotic. Fusidic acid binds with G factor in a molar ratio of 1 : 1 with an association constant 1.2×10^5 M^{-1}. The binding is strongly stimulated by ribosomes and GTP. Formation of fusidic acid - G factor - GDP - ribosome complex is demonstrated by equilibrium dialysis and ultracentrifugal separation methods. Measurements of binding at equilibrium indicate a stoichiometric combination of the four substances in a molar ratio of 1 : 1 : 1 : 1, provided that half of the ribosomes employed are active in this function. The association constant of fusidic acid is 2.2×10^6 M^{-1}. Less binding of the antibiotic is observed when fusidic acid-resistant G factor is used. A significant binding of fusidic acid is demonstrated when 70S ribosomes are replaced by 50S ribosomes or when GDP is used instead of GTP. Ki value for fusidic acid is 10^{-6} M in the GTPase reaction. It is in accordance with the association constant in the complex formation.

Fusidic acid inhibits the dissociation of fusidic acid - G factor - GDP - ribosome complex. The mechanism of action of fusidic acid is discussed and a model is presented for it.

Fusidic acid and related steroidal antibiotics have been shown to inhibit bacterial protein synthesis by interfering with the ribosome-dependent activity of G factor, *i. e.* hydrolysis of GTP to GDP and Pi, and translocation of peptidyl-tRNA from the acceptor site to the donor site on the ribosome[1~8]. In fusidic acid-resistant mutants, the resistance is associated with G factor but not with the ribosome[5,25]. BODLEY *et al.* have observed that fusidic acid facilitates the isolation of a ribosome - G factor - GDP complex[9]. They have demonstrated that fusidic acid inhibition of GTP hydrolysis results from the fact that it prevents the dissociation of a ribosome - G factor - GDP complex[10~11]. The chromosomal location of a fusidic acid resistant marker has been demonstrated to be at minute 64.2, about 0.2 minute from *str* A gene[12]. The antibiotic also inhibits protein synthesis in the mammalian system by preventing the ribosome-dependent GTPase activity of TF-II and translocation of peptidyl-tRNA[13~15]. By many investigators fusidic acid is used as a tool for studies on the mechanism of protein synthesis, particularly the translocation process. For the purpose of elucidating the mechanism of action of steroidal antibiotics, the binding of tritiated fusidic acid

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to G factor and/or ribosomes has been studied in the presence or absence of GTP. A part of the results has been presented in the previous communication[16].

Materials and Methods

Fusidic acid was generously given by W. O. GODTFREDSEN, Leo Pharmaceutical Products, Ballerup, Denmark. It was labelled with tritium by the method of WILZBACH; and purified by repeated crystallization from methanol and by thin-layer chromatography, using the solvent system (cyclohexane – chloroform – methanol – acetic acid, 10 : 80 : 2.5 : 10)[17]. The specific activity was 5×10^4 dpm/mμmole. It showed a single spot in a thin-layer chromatogram and no impurity was detected by chemical and biological assay.

Guanosine-8-^{14}C-5′-triphosphate (35 mCi/mmole) was a product of Radiochemical Centre, Amersham, England, and was diluted 23,000 cpm/mμmole with cold GTP. ^{32}P-γ-GTP was prepared by the method of CONWAY and LIPMANN[18], using photophosphorylation of GDP by spinach chloroplasts and purification by Dowex-1 chromatography.

The ribosomes were prepared by the method of LUCAS-LENARD[19] and G factor by the procedure of NISHIZUKA and LIPMANN[20] from *E. coli* B or a fusidic acid resistant mutant[5]. The specific activity of G factor from the sensitive organism was 16 units/mg, and that from the resistant one was 8 units/mg. One unit hydrolyzed 1 mμmole of GTP at 30°C for 10 minutes. One mg of pure G factor was reported to be 20 units, and the molecular weight to be 85,000[20,21]. It showed a single band in acrylamide gel electrophoresis. The ribosomes were thoroughly washed with buffer, containing 0.5 M NH_4Cl and 10 mM $Mg(AcO)_2$, and were free of GTPase activity. The amount of ribosomes was calculated on the basis that 1 mg was 14.4 OD_{260} and the molecular weight was 2,700,000.

The formation of fusidic acid – G factor – GDP – ribosome complex was studied by the ultracentrifugal separation method of HAYES and VELICK[22] in the mixture, containing ribosomes 1 mμmole (2.8 mg), G factor 1.4 mμmoles (2.24 units), ^{14}C-GTP 2 mμmoles, and ^{3}H-fusidic acid 2 mμmoles in 1 ml of buffer. The buffer consisted of 10 mM Tris-HCl, pH 7.8, 160 mM NH_4Cl, 10 mM Mg acetate, and 1 mM DTT. The concentration of fusidic acid or GTP were changed from 0.2 to 2.0 mμmoles/ml when indicated. The mixture without GTP was incubated at 30°C for 10 minutes and was further incubated for 10 minutes with GTP. It was transferred to a 2 ml tube and centrifuged for 30 minutes at 35,000 rpm, using Beckman 40 rotor. The top and bottom 0.2 ml fractions were collected and the content of the four factors was determined as described below. Equilibrium dialysis was performed according to methods previously described[23]. The mixture of ribosomes 1 mμmole, G factor 1.4 mμmoles and GTP 2 mμmoles in 0.5 ml of the buffer was incubated at 30°C for 10 minutes, placed in a bag (Visking cellulose tubing), and dialyzed against 2.5 ml buffer containing GTP 10 mμmoles and ^{3}H-fusidic acid 0.5~5.0 mμmoles with gentle rotation. The distribution of the antibiotic reached an equilibrium in 20 hours. The inner and outer liquids were assayed. The radioactivity of fusidic acid or nucleotide was determined in a liquid scintillation counter, using a dioxane base scintillator. The amount of ribosomes was estimated by UV absorption at 260 mμ and that of G factor by the GTP split reaction[20]. The latter was influenced by the presence of fusidic acid. Therefore the value was corrected by the standard inhibition curve.

Results

The Effect of Fusidic Acid on the Formation of G Factor-Guanosine Nucleotide-Ribosome Complex

Fusidic acid was observed to increase the binding of GTP with ribosomes and G factor (Table 1). When GTP was replaced by ^{32}P-γ-GTP, no significant radioactivity was detected in the ribosomal precipitate. It indicates that GTP was hydrolyzed to

Table 1. The effect of fusidic acid on the formation of G factor-guanosine nucleotide-ribosome complex.

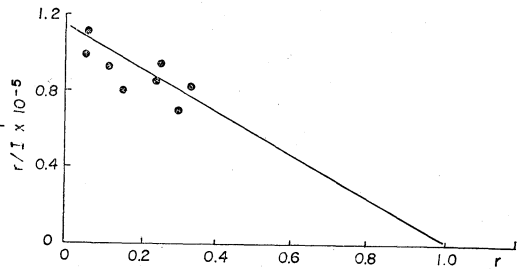
System	GTP bound cpm/OD_{260}
^{14}C-GTP, ribosomes	159
^{14}C-GTP, ribosomes, G factor	854
^{14}C-GTP, ribosomes, G factor, fusidic acid	4,180

The reaction mixture contained: ribosomes 1.5 μM, G factor 0.75 μM, ^{14}C-GTP 2.4 μM, Tris-Cl, pH 7.6, 20 mM, $Mg(AcO)_2$ 10 mM, NH_4Cl 10 mM, DTT 1 mM, fusidic acid 200 μM. It was incubated at 37°C for 5 minutes and centrifuged for 2 hours at 40,000 rpm. The radioactivity of the precipitate was determined.

Fig. 1. Scatchard plot for equilibrium binding of ^{3}H-fusidic acid to G factor.

r : $\frac{\text{m}\mu\text{moles fusidic acid bound}}{\text{m}\mu\text{moles G factor}}$

I : free fusidic acid (M)



GDP in the complex in the presence or absence of fusidic acid.

The Binding of ^{3}H-Fusidic Acid to Ribosomes, G Factor and/or Guanosine Nucleotide

Fusidic acid was demonstrated by equilibrium dialysis to bind to G factor in a molar ratio of 1:1 with an association constant 1.2×10^{5} M^{-1} (Fig. 1). Less binding was observed with G factor obtained from the fusidic acid-resistant mutant. The binding of the sensitive factor was markedly enhanced by the presence of ribosomes and GTP. A relatively stable complex of the four substances was demonstrated by the ultracentrifugal separation method. The binding of fusidic acid to the complex was much reduced, when any of the other three factors was omitted in the reaction mixture or G factor was replaced by the one resistant to the antibiotic. A significant binding of fusidic acid was also observed when GTP was replaced by GDP or when 70S ribosomes were replaced by 50S ribosomes (Table 2).

Table 2. Binding of ^{3}H-fusidic acid to ribosomes, G factor and/or guanosine nucleotide.

System	^{3}H-Fusidic acid bound	Method
G factor, GTP, 70S ribosomes	100. %	ED, US
G factor, GDP, 70S ribosomes	52.3	US
G factor, GTP, 50S sibosomes	57.6	US
G factor, GTP, 30S ribosomes	15.7	US
G factor	9.2	ED
Fusidic acid-resistant G factor	3.2	ED
70S ribosomes	0	US
G factor, GTP	14.1	ED
70S ribosomes, GTP	0	US
G factor, 70S ribosomes	7.7	US
Fusidic acid-resistant G factor, GTP, 70S ribosomes	11.2	US

ED: Equilibrium dialysis. 100 % = 0.27 mμmoles/0.5 ml of reaction mixture inside the dialysis bag.
US: Ultracentrifugal separation. 100 % = 0.29 mμmoles/0.2 ml of the bottom fraction.

For the purpose of determining the association constants and numbers of binding sites in the quartet complex, the ultracentrifugal separation was performed with various concentrations of fusidic acid, or GTP. The results were plotted according to the SCATCHARD equation for equilibrium binding[23]. As illustrated in Figs. 2 and 3, the maximal bindind ratio of fusidic acid to the ribosome was 0.5. The association

Fig. 2. Scatchard plot for equilibrium binding of ^{3}H-fusidic acid to ribosomes in the presence of G factor and GTP.
r : $\frac{\text{m}\mu\text{moles fusidic acid bound}}{\text{m}\mu\text{mole ribosome}}$
I : free fusidic acid (M)

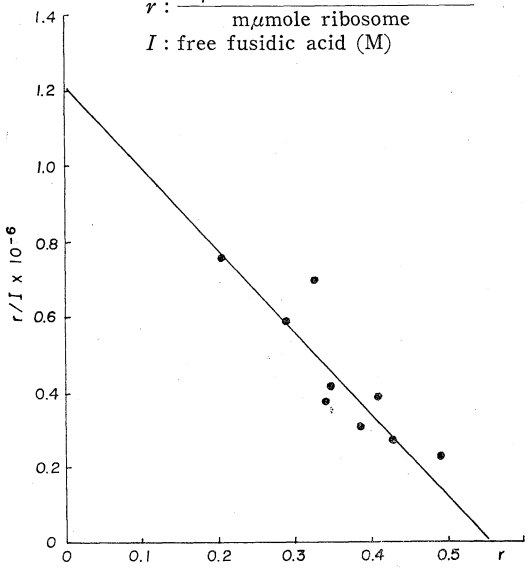


Fig. 3. Scatchard plot for equilibrium binding of ^{3}H-fusidic acid to ribosomes in the presence of G factor and GDP.
r : $\frac{\text{m}\mu\text{moles fusidic acid bound}}{\text{m}\mu\text{mole ribosome}}$
I : free fusidic acid (M)

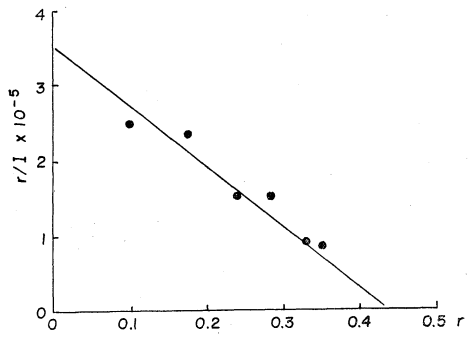
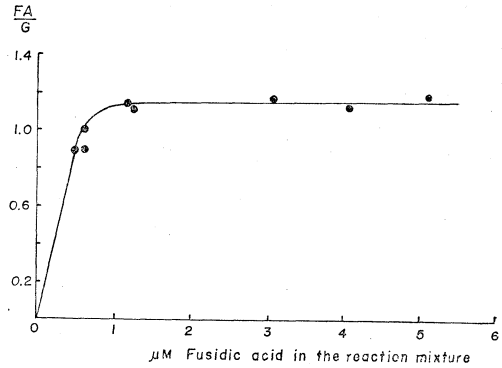


Fig. 4. Binding of fusidic acid and G factor to ribosomes in the presence of GTP.
$\frac{FA}{G}$: $\frac{\text{m}\mu\text{moles fusidic acid bound}}{\text{m}\mu\text{moles G factor bound}}$



constant of fusidic acid was 2.2×10^{6} M^{-1} with GTP and 0.8×10^{6} M^{-1} with GDP. The maximal binding ratio of guanosine nucleotide to the ribosome was 0.5.

The association constant of guanosine nucleotide was 1.6×10^{5} M^{-1} in the absence of fusidic acid and it was increased by the presence of the antibiotic. The affinity of guanosine nucleotide was much reduced, when G factor was replaced by the one resistant to fusidic acid (Table 3). The molar raito of fusidic acid and G factor in the complex was approximately 1 : 1 in a range of 1~5 μM fusidic acid (Fig. 4).

It was concluded that the binding of 1 mμmole fusidic acid required 1 mμmole guanosine nucleotide, 1 mμmole G factor and *ca.* 2 mμmoles ribosomes. Assuming that about half of the ribosomes employed are active in this function, the results indicate a stoichiometric combination of the four substances in a molar ratio of 1 : 1 : 1 : 1.

Table 3. Binding of ^{14}C-GTP with G factor and ribosomes in the presence and absence of fusidic acid.

Additions		Binding of ^{14}C-GTP	
		maximal binding ratio to ribosomes	Association constant
G factor, ribosomes		0.5	1.6×10^{5} M^{-1}
G factor, ribosomes, fusidic acid	2 μM	0.5	1.8×10^{5}
	10 μM	0.5	3.2×10^{5}
G factor (fusidic acid-resistant), ribosomes		0.5	0.7×10^{5}

Table 4. Dissociation of ^{14}C-GDP from the G factor-GDP-ribosome complex in the presence of GTP.

Additions	^{14}C-GDP in the complex (cpm/OD_{260})
None	213
GTP	107
GTP, fusidic acid 200 μM	356

The reaction mixture contained: ribosomes 1.5 μM, G factor 0.75 μM, ^{14}C-GTP 24 μM, Tris-Cl, pH 7.6, 20 mM, $Mg(AcO)_2$ 10 mM, NH_4Cl 10 mM, DTT 1 mM. It was incubated at 30℃ for 5 minutes and centrifuged for 2 hours at 40,000 rpm. The precipitate was dissolved in 3.5 ml of the same buffer and cold GTP was added at the concentration of 2.8 mμmoles/ml. The radioactivity was 854 cpm/OD_{260}. It was further incubated at 30℃ for 5 minutes and centrifuged for 2 hours at 40,000 rpm. The radioactivity of the precipitate was determined.

Inhibition by Fusidic Acid of Dissociation of G Factor-GDP-Ribosome Complex

Addition of GTP to the G factor - ^{14}C - GDP - ribosome complex increased the dissociation of the complex. Fusidic acid was observed to prevent the dissociation, presumably by binding to the complex (Table 4). The incorporation of ^{14}C-GTP into the complex was compared when it was introduced into the reaction mixture at the beginning of incubation or at 30 minutes. In the absence of fusidic acid no difference was observed between the two cases, indicating an equilibrium of the incorporation of ^{14}C-GTP. However, in the presence of fusidic acid, a significant difference was demonstrated. It indicates that the dissociation of fusidic acid - G factor - GDP - ribosome complex are more difficult than that of G factor - GDP - ribosome complex (Table 5).

Effects of Fusidic Acid on Ribosome-Dependent GTPase Activity of G Factor

This activity was inhibited by fusidic acid: Ki

Table 5. Incorporation of ^{14}C-GTP into the G factor-GDP-ribosome complex.

Incubation period of ^{14}C-GTP	^{14}C-GTP incorporated (cpm/OD_{260})	
	+ fusidic	− fusidic
60 min.	165	102
30 min.	120	102

The reaction mixture contained: ribosomes 0.5 μM, G factor 1 μM, cold GTP 10 μM, Tris-Cl, pH 7.6, 20 mM, $Mg(AcO)_2$ 10 mM, NH_4Cl 10 mM and DTT 1 mM, with or without fusidic acid 5 μM. It was incubated at 30℃ for 60 minutes. ^{14}C-GTP was added to the mixture at the beginning of the incubation or 30 minutes afterwards. After centrifugation, the ribosomal pellets were assayed for radioactivity.

Fig. 5. Hofstee plot for GTP-cleavage reaction by G factor and ribosomes.

The reaction mixture contained: ribosomes 200 μg, G factor 5 μg, ^{32}P-γ-GTP 5 to 160 mμmoles, Tris-Cl, pH 7.6, 20 mM, $Mg(AcO)_2$ 10 mM, NH_4Cl 10 mM and DTT 1 mM, with or without fusidic acid 2 μM. It was incubated for 10 minutes at 30℃, and assayed by the method of NISHIZUKA and LIPMANN[20].

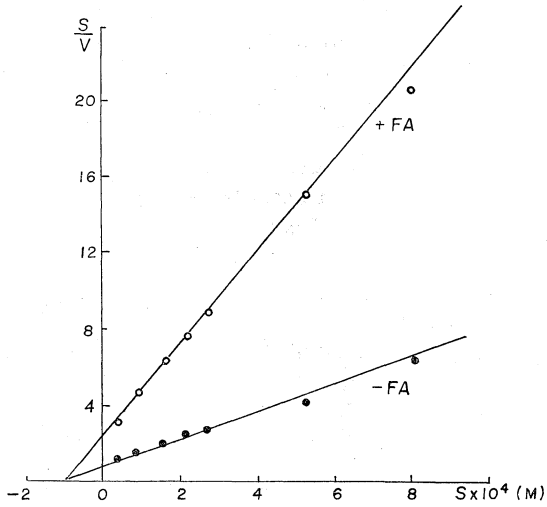
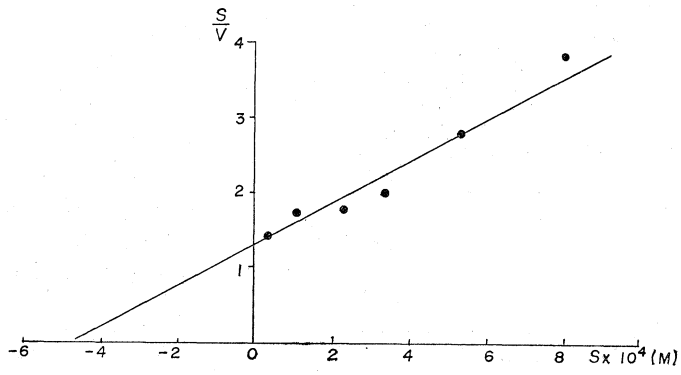


Fig. 6. Hofstee plot for GTP-cleavage reaction by ribosomes and G factor from a fusidic acid resistant mutant.

The assay procedure was the same as described in the legend of Fig. 5.

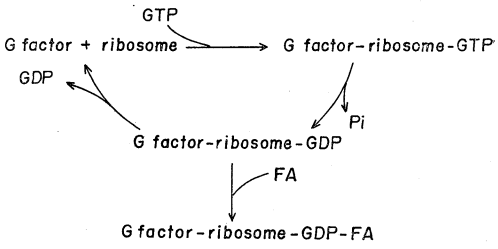


was 10^{-6} M and Km for GTP was 8.5×10^{-5} M (Fig. 5). It was in accordance with the association constant of fusidic acid to the complex, indicating that the complex formation may be the basis of the effect of fusidic acid. As described above, a less affinity of fusidic acid was observed when G factor is replaced by the one obtained from the resistant mutant. G factor from the resistant mutant showed Km value of 4.8×10^{-4} M for GTP in the GTPase reaction which was about five times higher than that of the sensitive G factor (Fig. 6).

Discussion

Formation of fusidic acid - G factor - GDP - ribosome complex in a molar ratio of 1 : 1 : 1 : 1 has been demonstrated in the present experiments. GTP is hydrolyzed to GDP in the complex in the presence and absence of fusidic acid. This shows that fusidic acid does not inhibit the GTPase reaction *per se*. Fusidic acid interferes with the dissociation of G factor - GDP - ribosome complex. The results indicate that the inhibition by fusidic acid of the GTPase reaction is due to the inhibition of dissociation of the complex in the cyclic reaction as presented in Fig. 7.

Fig. 7. Diagrammatic representation of mechanism of fusidic acid inhibition of the GTPase reaction.



The complex formation of the four substances is also observed when 70S ribosome are replaced by 50S ribosomes. It suggests that G factor interacts with the 50S ribosomal subunit. The assumption is in accordance with the proposal of Bodley and Lin[24].

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