A RESOLUTION OF CONFLICTING REPORTS CONCERNING THE MODE OF ACTION OF FUSIDIC ACID

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1. Introduction

During protein synthesis peptidyl-tRNA is translocated from the ribosomal A site to the P site in a reaction requiring the protein elongation factor G (EF-G) and hydrolysis of one molecule of GTP [1, 2]. Although fusidic acid was originally found to inhibit both GTP hydrolysis [3] and translocation-dependent reactions in vitro [3–5] it was later demonstrated that one round of GTP hydrolysis could occur in the presence of the drug [6]. The resultant ribosome–EF-G–GDP complexes [7] were, however, stabilized and it was suggested that fusidic acid inhibits protein synthesis by preventing dissociation of these complexes, implying an inhibition of translocation. However, there have recently been reports that fusidic acid blocked peptidyl-tRNA in the ribosomal P site, both in vivo [8] and in vitro [9] in contrast to the expected behaviour if translocation were inhibited by the antibiotic.

The aim of the present experiments was to reconcile the apparent contradictions between the earlier evidence and the more recent work on the mode of action of fusidic acid. Our results show that while under certain conditions fusidic acid *can* slow down translocation in vitro (without completely inhibiting this process) this inhibitory effect is misleading since it is closely dependent on the concentration of EF-G used in the incubation mixture. Indeed, when a molar excess of EF-G, relative to ribosomes, is employed fusidic acid neither inhibits the rate nor the extent of translocation and instead blocks peptidyl-tRNA in the ribosomal P site as suggested recently [8, 9].

2. Materials and methods

Escherichia coli (strain B163) was grown to late logarithmic phase, rapidly chilled, harvested and the cells stored frozen until use [10]. Crude extracts were prepared [11] and adjusted to a ribosome concentration of 10 mg/ml. ^{14}C-labelled amino acids were incorporated into peptidyl-tRNA as directed by the endogenous mRNA of the extract and as described previously [11].

Ribosome-free supernatant fraction was obtained from crude extracts by centrifugation [11] and was dialysed for 18 hr at 4°C against 5 mM Tris–Cl buffer, pH 7.4, containing 10 mM magnesium acetate, 86 mM potassium chloride and 6 mM 2-mercaptoethanol. The supernatant fraction was stoed in small aliquots at −20°C and was used as an unpurified source of EF-G.

2.1. *Translocation studies*

For translocation studies incorporation mixtures were diluted with 5 mM Tris–Cl buffer, pH 7.4, containing 10 mM magnesium acetate, 0.5 M ammonium chloride and 15% (w/v) sucrose and ribosomes were pelletted by centrifugation at 165 000 *g* for 1 hr. Ribosome pellets were resuspended and the washing

procedure repeated three times to remove all traces of bound EF-G. Ribosomes were finally resuspended to a concentration of 3 mg/ml in 5 mM Tris–Cl buffer, pH 7.4, containing 10 mM magnesium acetate and 86 mM potassium chloride. Samples were incubated with puromycin (0.1 mM) and other components as described in the figure legends and release from ribosomes of ^{14}C-labelled peptide estimated as previously described [11, 12].

2.2. *Studies on the distribution of peptidyl-tRNA between ribosomal binding sites*

Ribosomes were isolated from crude extracts of *E. coli* actively synthesizing peptide. One incubation mixture was inhibited with 3 mM fusidic acid and a second sample inhibited with chlortetracycline (100 μg/ml). Ribosomes were prepared by dilution of incubation mixtures with 5 mM Tris–Cl buffer, pH 7.4, containing 10 mM magnesium acetate and 86 mM potassium chloride, followed by centrifugation as in 2.1 above. Release of peptide by puromycin was determined as described under section 2.1.

Purified EF-G was a generous gift from Dr. J.W. Bodley. Sodium fusidate was obtained from Leo Laboratories Ltd. ^{14}C-labelled *Chlorella* protein hydrolysate (52 mCi/matom carbon) was obtained from the Radiochemical Centre (Amersham, England). Puromycin dihydrochloride was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, USA.

3. Results and discussion

Release of nascent peptides from isolated, washed ribosomes by puromycin is used as an assay for the peptidyl transferase enzyme on the 50 S ribosomal sununit. In the absence of translocation, only peptidyl-tRNA bound to the ribosomal P site can react with puromycin. Release of peptide can be followed as a decrease in ribosome-associated ^{14}C- counts.

In control incubations from which supernatant fraction and GTP were omitted from ribosome suspensions approximately 35% of the total ribosome-bound radioactive peptide was released (from the ribosomal P site) over a 10 min incubation period (fig. 1). Where ribosomes were incubated with GTP

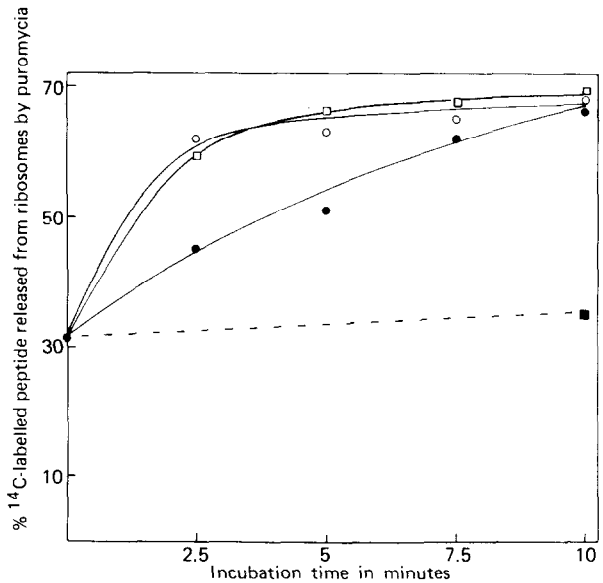

Fig. 1. Effect of fusidic acid on the puromycin reaction with washed ribosomes in the presence of GTP and either supernatant fraction or purified EF-G. Labelled ribosome suspensions (specific activity 20 000 dpm/mg) were prepared as described in section 2.1. Samples in a final volume of 1 ml contained ribosomes (1.5 mg), ATP (1 μmole), phosphoenolpyruvate (5 μmole), GTP (0.3 μmole), pyruvate kinase (50 μg), supernatant fraction and puromycin (0.1 mM). For experiments using purified EF-G ATP, phosphoenolpyruvate and pyruvate kinase were omitted and GTP (0.15 μmole) added. Incubations at 30°C were in the presence or absence of fuside acid (0.3 mM). Samples were chilled on ice, ribosomes isolated and release of nascent protein determined as described in section 2.1. (○) Control ribosomes incubated as above in the presence of either supernatant fraction (0.02 ml or 0.2 ml) or purified EF-G (0.08 μmole or 0.8 μmole). (●) Fusidic acid present along with supernatant fraction (0.02 ml) or purified EF-G (0.08 μmole). (□) Fusidic acid present along with supernatant fraction (0.2 ml) or purified EF-G (0.8 μmole). (■) Control ribosomes incubated with puromycin alone.

and either supernatant fraction or purified EF-G (fig. 1) those peptides (30–35% of the total) which were released promptly, even at 0°C, were assumed to have been bound in the ribosomal P site as peptidyl-tRNA. A further 35% of the radioactive peptides were released over a 5 min incubation in a reaction dependent upon translocation of peptidyl-tRNA from the ribosomal A site to the P site. Addition of 0.3 mM fusidic acid causes a considerable slowing of

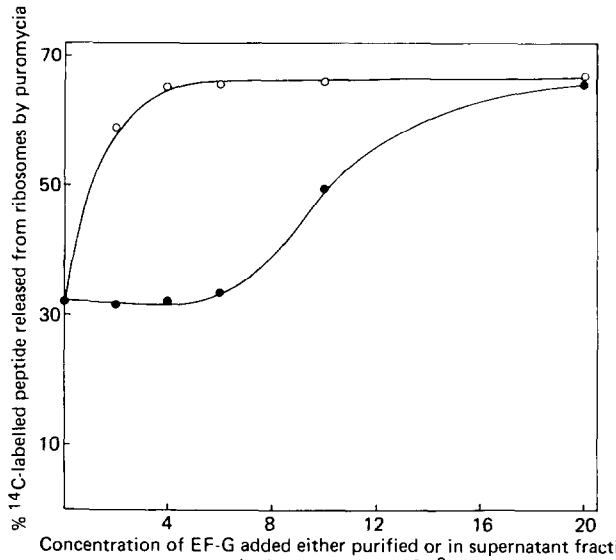

Fig. 2. Effect of fusidic acid on the puromycin reaction with washed ribosomes in the presence of GTP and different concentrations of supernatant fraction or purified EF-G. Conditions for this experiment are described in the legend to fig. 1 except that different amounts of either supernatant fraction or EF-G were added as indicated. Incubation was at 30°C for 2.5 min; Release of nascent protein from ribosomes was determined as under fig. 1. Results with either supernatant fraction or purified EF-G were identical: (○) control; (●) fusidic acid present.

Table 1
Inhibition of ribosomes in the puromycin reaction by pretreatment of crude extracts with either fusidic acid or chlortetracycline during protein synthesis

System	Protein released from ribosomes by puromycin (%)
(a) Control ribosomes	36
(b) Ribosomes from extracts treated with fusidic acid	45
(c) Ribosomes from extracts treated with chlortetracycline	54
(d) Control ribosomes incubated with GTP	67

Incorporation mixtures were incubated for 5 min at 30°C. Two control samples (1.5 ml each) were chilled on ice and to two other samples (1.5 ml) was added fusidic acid (3 mM) or chlortetracycline (100 μg/ml). Incubation was continued for 5 min before chilling. Ribosomes were isolated as described in Materials and methods and after resuspension 1 ml samples (a), (b) and (c) were incubated with puromycin (0.1 mM) alone for 5 min. Sample (d) was incubated with puromycin (0.1 mM) in addition to a reaction mixture (1 ml) containing phosphoenolpyruvate (5 μmole), ATP (1 μmole), GTP (0.15 μmole), pyruvate kinase (50 μg), 2-mercaptoethanol (6 μmole) and supernatant fraction (0.2 ml). The amount of peptide released from ribosomes by puromycin was determined as described under fig. 1.

this translocation-dependent release when limiting amounts of EF-G are used – conditions which approximate those used previously by others (3–5). However, in *E. coli* extracts prepared as in the present work, only approximately 10% of the ribosomes are 'active' in protein synthesis [13]. In the experiments presented in fig. 1 EF-G, whether added as purified factor or as crude supernatant fraction, was present either in a slight molar excess with respect to the 'active' ribosomes or in 10-fold greater concentration, assuming the values for EF-G content of *E. coli* calculated by Gordon [14]. In all cases fusidic acid was present in considerable molar excess with respect to other components. As seen in fig. 1 whether or not fusidic acid inhibited translocation was closely dependent upon the relative concentrations of EF-G and ribosomes.

It has been suggested [8, 15] that sequestration of EF-G on inactive ribosomes might account, at least in part, for the inhibition of translocation by fusidic acid as reported by others [3–5]. Such sequestration would reduce the amount of EF-G available to 'active' ribosomes particularly if the levels of EF-G employed were limiting. The data in fig. 1 support such an interpretation as does the data of fig. 2 where the effect of fusidic acid on one round of translocation was investigated in the presence of a range of EF-G concentrations. Increasing the concentration of the factor overcomes the inhibition of translocation which can be caused by fusidic acid. This observation correlates well with the report by Kaji et al. [16] that EF-G dependent release of deacylated-tRNA from ribosomes – an event normally associated with translocation – is insensitive to fusidic acid when an excess of EF-G is employed. Both their observations [16] and ours are consistent with the sequestration theory as outlined above. In our experiments we note that in the absence of

fusidic acid the system reaches saturation with EF-G when this factor and the 'active' ribosomes are present in approximately equimolar concentrations. The presence of 0.3 mM fusidic acid increases four or five-fold the concentration of EF-G necessary for maximal release of peptide.

In our opinion these results reconcile apparent contradictions in the literature and establish the sequestration hypothesis [8, 15] as the underlying cause of this discrepancy. We stress that any inhibitory action of fusidic acid upon translocation, expressed in vitro, is probably artificial since in intact bacteria elongation factors for protein synthesis are apparently not present in limiting quantity [14] and it is most improbable that growing cells normally contain a large proportion of inactive ribosomes.

Finally, we studied the effect of fusidic acid in a complete protein synthesizing system – a crude extract of *E. coli* supplemented with a mixture of ^{14}C-labelled amino acids together with an energy-generating source [12]. This system utilises endogenous mRNA. After treatment of such a system with fusidic acid, ribosomes were isolated and assayed for reactivity towards puromycin. The results are presented in table 1. Following incubation with fusidic acid the distribution of peptidyl-tRNA between ribosomal sites is altered in such a way that the amount of material capable of reacting with puromycin, in the absence of added GTP and supernatant fraction is increased. For comparison, the effect of chlortetracycline in an identical system is also shown (table 1c). Chlortetracycline prevents aminoacyl-tRNA binding at the ribosomal A site and peptidyl-tRNA is blocked in the ribosomal P site resulting in an increase in puromycin reactivity [12]. The total amount of peptidyl-tRNA which is potentially capable of reacting with puromycin on ribosomes isolated from an uninhibited system is given in table 1 (d) where incubation of such ribosomes with GTP and supernatant fraction allows translocation. In this case peptidyl-tRNA present in both ribosomal A and P sites at the time of removal from the complete system reacts subsequently with puromycin. The data in table 1 indicate that both fusidic acid and chlortetracycline produce similar results in the system with a more pronounced effect for the latter antibiotic. Furthermore, the results do not suggest that fusidic acid, employed as above in a complete cell-free system, inhibits translocation in vitro since such an inhibition would block peptidyl-tRNA in the ribosomal A site [12] and would allow less reaction with puromycin, as previously observed with erythromycin [12].

This result can be rationalized according to the single 'GTPase' model of ribosome function in which EF-G and elongation factor Tu (EF-Tu) interact alternately with a common ribosomal site (or in mutually exclusive fashion with two adjacent sites) in or near the A site moiety of the 50 S ribosomal subunit [17, 18]. A complex of EF-G and GDP on this 'GTPase' centre, stabilized by fusidic acid, would inhibit not translocation but rather the next essential function of that site which would be an interaction with an (aminoacyl-tRNA–EF-Tu–GTP) complex. Such an inhibitory effect would be expected to block peptidyl-tRNA in the ribosomal P site [8] since fusidic acid would primarily inhibit the binding of aminoacyl-tRNA into the ribosomal A site after a translocation event had occurred.

Although our data are consistent with this interpretation, the blockage caused by fusidic acid is clearly not complete and our observation may be more in line with the long term effects obtained with this drug in vivo [19]. Thus, while fusidic acid initially confines peptidyl-tRNA to the ribosomal P site, a slow cycle is eventually established and peptidyl-tRNA enters and leaves both sites. According to this model fusidic acid can hold peptidyl-tRNA in either ribosomal site, although less efficiently in the A than in the P site – consistent with a secondary effect of fusidic acid possibly on translocation itself. We do not know if this effect is related to the sequestration of EF-G discussed earlier although we note that the presence of EF-G and GDP on the 'GTPase' site might inhibit translocation by excluding GTP. Such a situation might arise at unusually high GDP/GTP ratios. Alternative explanations might be connected with a recent report [20] that fusidic acid can affect both ribosomal A and P sites under particular conditions.

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