RIBOSOMAL SITES INVOLVED IN BINDING OF AMINOACYL-tRNA AND EF 2. MODE OF ACTION OF FUSIDIC ACID

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1. Introduction

The antibiotic fusidic acid has been found to block protein synthesis in bacterial [1–3] and in eukaryotic systems [4, 5]. It was postulated that fusidic acid inhibits protein synthesis by blocking ribosomal translocation since it inhibits (a) the GTP hydrolysis dependent on ribosomes and either elongation factor G (EF G) (in bacterial systems) or elongation factor 2 (EF 2) (in eukaryotic systems) and, under appropriate conditions, (b) the GTP plus elongation factor-dependent reaction of Ac-Phe-tRNA or Phe-tRNA with puromycin [1–7]. Moreover, a modified EF G resistant to fusidic acid has been described in bacterial mutants resistant to the antibiotic [8]. Further studies have shown that fusidic acid stabilizes a GDP–ribosome–EF G (or EF 2) complex [9, 10]; it was thought that formation of this complex [11] caused the inhibition of translocation. There is, however, increasing evidence in bacterial systems that the primary effect of fusidic acid in protein synthesis is not the inhibition of translocation: for fusidic acid-inhibited polysomes, formed on synthetic (poly (U) and viral R17-RNA), or endogenous (*Escherichia coli* or *Bacillus subtilis*) messenger have their nascent peptides in the puromycin-reactive position [12–15]. It has also been shown that formation of the complexes (i) GDP–ribosome–fusidic acid–EF G or (ii) EF G–ribosome–GDPCP (5′-guanylylmethylene diphosphonate) [16–19] inhibits aminoacyl-tRNA binding to the A-site. Thus, these results suggest that in bacterial systems due to formation of the complex GDP–ribosome–fusidic acid–EF G the antibiotic inhibits protein synthesis by blocking aminoacyl-tRNA binding to the A-site.

Our recent studies with human tonsil ribosomes suggest a similar action of fusidic acid in eukaryotic systems, since the antibiotic does not inhibit translocation of Ac-Phe-tRNA and Phe-tRNA bound to the ribosomal A-site, as measured by the puromycin reaction after incubation with EF 2 and GTP [20]. We have now continued the studies with this system to help to clarify (a) the mode of action of fusidic acid and (b) the relationship between the ribosomal sites implicated in binding of EF 2 and aminoacyl-tRNA. The results obtained are presented in this paper.

2. Methods

Sources of reagents and methods used to prepare and Ac-[^{3}H]Phe-tRNA (18 Ci/mmole), [^{14}C]Phe- and Ac-[^{14}C]Phe-tRNA (500 mCi/mmole), human tonsil ribosomes and elongation factors EF 1 and EF 2 and to measure binding of Phe-tRNA and Ac-Phe-tRNA to the ribosomal A-site, translocation of Ac-Phe-tRNA and poly (U)-directed [^{14}C]phenylalanine incorporation, were essentially as previously described [18, 20–22].

3. Results

3.1. Effect of fusidic acid on polyphenylalanine synthesis at different concentrations of EF 2

The effect of fusidic acid (10^{-4} M) on poly (U)-directed [^{14}C]phenylalanine incorporation at different EF 2 concentrations is shown in fig. 1. It is seen that the inhibitory effect of the antibiotic decreases when the

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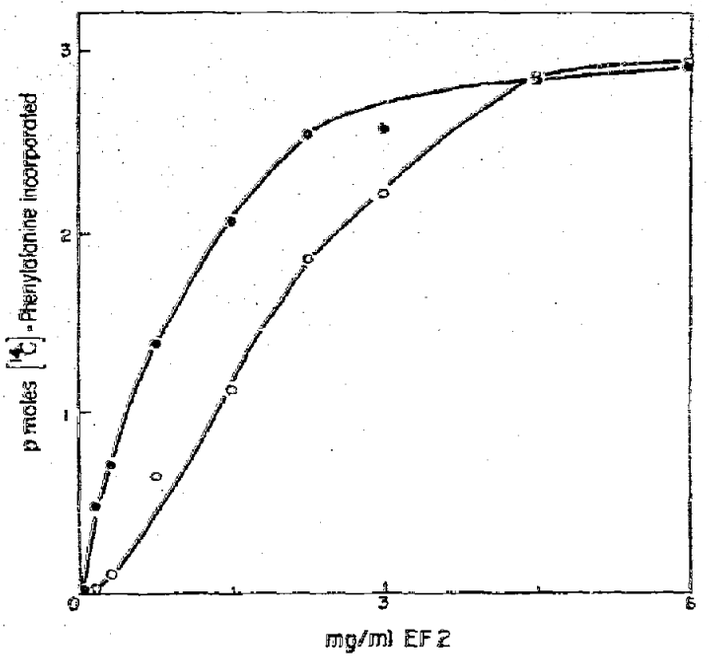

Fig. 1. Synthesis of poly(U)-directed polyphenylalanine. Effect of fusidic acid at different concentrations of EF 2. Complete reaction mixtures contained, in 0.1 ml, 50 mM Tris-HCl (pH 7.4), 11 mM magnesium chloride, 60 mM potassium chloride, 8 mM 2-mercaptoethanol, 2 mM GTP, 100 μg/ml poly(U), 0.06 μM [^{14}C]Phe-tRNA, 0.3 μM ribosomes, 5.4 mg/ml of EF 1 and the required concentrations of EF 2. Purity of EF 2 was approx. 50% [26]. The reaction mixtures were incubated at 37° for 20 min. All values were corrected by subtraction of blanks without elongation factors (0.14 pmoles). (●—●—●) Control; (○—○—○) with 10^{-4} M fusidic acid.

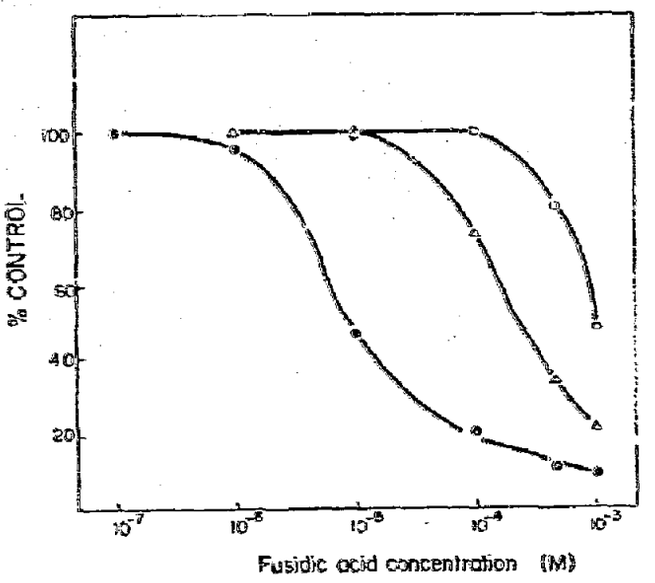

Fig. 2. Effect of fusidic acid on synthesis of poly(U)-directed polyphenylalanine. Dependence of EF 2 concentration. Reaction mixtures and incubations were as indicated under fig. 1. Additions of EF 2 were either 0.15 mg/ml (●—●—●), or 1.5 mg/ml (△—△—△), or 6.0 mg/ml (○—○—○).

ratio of EF 2 to ribosomes increases and it is abolished at saturating amounts of EF 2. The effect of increasing the concentration of fusidic acid at three different ratios of EF 2 to ribosomes is shown in fig. 2. Higher concentrations of the antibiotic were required for inhibition when the concentration of EF 2 was increased.

3.2. *Effect of fusidic acid on translocation of Ac-Phe-tRNA*

The effect of fusidic acid on translocation was studied by measuring the extent of the GTP- and EF 2-dependent reaction of Ac-Phe-tRNA prebound to the A-site with puromycin. Fig. 3 shows that there is a clear inhibition of translocation by fusidic acid at low concentrations of EF 2, but it decreases and is even abolished when the concentration of the factor is increased. The effect of fusidic acid is also dependent on the ribosome concentration since the inhibition observed at low ratios of EF 2 to ribosomes is increased and can be complete when a sufficient amount of free ribosomes is present in the reaction mixture (table 1). This finding supports previous reports suggesting that the inhibitory effect of fusidic acid is due to the sequestering of EF 2 molecules in the form of stable GDP—ribosome—EF 2—fusidic acid complexes [17, 18].

3.3. *Ac-Phe-tRNA and Phe-tRNA binding to ribosomes pretreated with EF 2*

It has recently been shown that the EF 2–GTP complex interacts specifically with the large ribosome subunit [23]. To investigate the possible relation between its interaction site and the ribosomal A-site we have formed the complexes (a) GDP–EF 2–ribosome, (b) GDPCP–EF 2–ribosome and (c) GDP–EF 2–fusidic acid–ribosome and studied their ability to bind acylated tRNA to the A-site. For this purpose we have measured the binding of Phe-tRNA, either enzymic or non-enzymic at low and high Mg^{2+} concentrations respectively under conditions promoting binding mostly to the A-site [20]. We have also measured the non-enzymic binding of Ac-Phe-tRNA at high Mg^{2+} concentration and

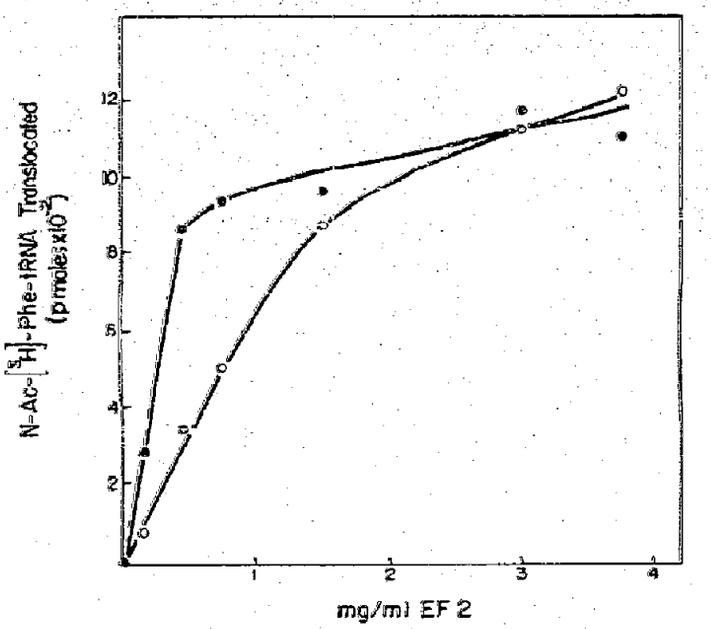

Fig. 3. Effect of fusidic acid on translocation of Ac-[^{3}H]Phe-tRNA. Dependence on EF 2 concentration. Reaction mixtures containing, in 0.5 ml, 50 mM Tris-HCl (pH 7.4), 15 mM magnesium chloride, 60 mM potassium chloride, 0.4 mg/ml poly(U), 1.5 μM ribosomes and 0.12 μM Ac-[^{3}H]Phe-tRNA were incubated at 37° for 30 min. The Ac-[^{3}H]Phe-tRNA–ribosome complex was separated by centrifugation [20], resuspended and taken for the translocation reaction in mixtures containing finally, in 0.1 ml, 50 mM Tris-HCl (pH 7.4), 11 mM magnesium chloride, 60 mM potassium chloride, 8 mM 2-mercaptoethanol, 1 mM GTP, 0.0023 μM Ac-[^{3}H]Phe-tRNA–ribosome complex, EF 2 as indicated and 1 ml puromycin. Incubations were carried out at 37° for 5 min. Ac-[^{3}H]Phe-puromycin formed was extracted with ethyl acetate and radioactivity estimated [27]. All values were corrected by subtraction of blanks without EF 2 and GTP (0.04 pmoles). (•—•—•) Control; (o—o—o) with 10^{-3} M fusidic acid.

under conditions leading to approximately the same amount of Ac-Phe-tRNA bound to the A- and P-sites [20]. Table 2 shows, with two different preparations of ribosomes ((a) and (b)), that EF 2 partially inhibited binding of both substrates to the A-site. The inhibition increased in the presence of GTP and was even higher when fusidic acid was added (this should be expected since the EF 2–ribosome complex is more stable in the presence of fusidic acid, [10]). The highest inhibition was obtained when GTP was replaced by its nonhydrolyzable analogue. The results were very similar with either enzymically or non-enzymically bound Phe-tRNA. Inhibition however, was similar with Ac-Phe-tRNA but in some cases (as in experiment (a)) it was higher than 50%. Since only about 50% of the Ac-Phe-tRNA binds to the A-site [20] these data suggest that EF 2 also inhibits to some extent the binding to the ribosomal P-site. Essentially similar results were obtained by other workers studying Phe-tRNA binding when either the complex GDPCP–EF 2–ribosome [24] or GDP–EF 2–ribosome–fusidic acid [25] were formed.

4. Discussion

Fusidic acid forms a stable complex GDP–ribosome–fusidic acid–EF 2 [10]. Inhibition of protein synthesis by fusidic acid appears to be a consequence of its effect sequestering all the EF 2 available due to the formation of the GDP–ribosome–fusidic acid–EF 2 complex. Our results support this hypothesis since the effect of fusidic acid in cell-free systems can be decreased or

Table 1
Effect of fusidic acid on translocation of Ac-[^{3}H]Phe-tRNA; dependence on the concentration of ribosomes.

Additions to the system	Ac-[^{3}H]Phe-puromycin formation (pmoles)	Translocation (% control)
None	0.150	100
+ Fusidic acid	0.048	32
+ 0.5 μM free ribosomes	0.120	100
+ 0.5 μM free ribosomes + fusidic acid	0.022	18
+ 2.0 μM free ribosomes	0.106	100
+ 2.0 μM free ribosomes + fusidic acid	0.001	1

Experimental conditions were as indicated under fig. 3 but 0.0047 μM Ac-[^{3}H]Phe-tRNA–ribosome complex and 2.3 mg/ml EF 2 were added in all cases. 10^{-3} M fusidic acid was added when required. Free ribosomes were added when indicated to the reaction mixture prior to the translocation reaction. All values were corrected by subtraction of blanks without EF 2 + GTP (0.068 pmoles).

Table 2
Binding of [^{14}C]Phe- and N-Ac-[^{14}C]Phe-tRNA binding to ribosomes; effect of prior addition of EF 2.

Additions to the system	Enzymic binding of [^{14}C]Phe-tRNA (% control)		Non-enzymic binding of [^{14}C]Phe-tRNA (% control)		Non-enzymic binding of Ac-[^{14}C]Phe-tRNA (% control)	
	Experiment (a)	(b)	Experiment (a)	(b)	Experiment (a)	(b)
None	100	100	100	100	100	100
+ EF 2	–	–	63	45	42	76
+ GTP	–	–	92	72	92	103
+ Fusidic acid	59	76	101	91	92	100
+ GDPCP	60	66	76	56	71	69
+ EF 2 + GTP	–	–	36	34	29	65
+ EF 2 + GTP + fusidic acid	21	11	9	6	16	44
+ EF 2 + GDPCP	4	3	1	0	14	43

Reactions for non-enzymic binding of [^{14}C]Phe- and Ac-[^{14}C]Phe-tRNA and enzymic binding of [^{14}C]Phe-tRNA were carried out as previously described [20]. Prior to these reactions 0.29 μM ribosomes were preincubated at 37° for 5 min with 7.8 mg/ml of EF 2 or 0.2 mM GTP or 10^{-3} M fusidic acid or 0.2 mM GDPCP when indicated. 2.8 mg/ml of EF 1 or 0.042 μM [^{14}C]Phe-tRNA or 0.023 μM N-Ac-[^{14}C]Phe-tRNA were then added when required and incubations carried out at 37° for 20 min [20]. Two different batches of ribosomes were used for experiments (a) and (b). In the control experiments carried out with the complete reaction mixtures, without any further addition, enzymic binding of [^{14}C]Phe-tRNA was 1.10 and 3.26 pmoles in experiments (a) and (b) respectively, non-enzymic binding of [^{14}C]Phe-tRNA was 1.20 and 2.67 pmoles and non-enzymic binding of Ac-[^{14}C]Phe-tRNA was 0.62 and 0.98 pmoles. Of the different ribosome preparations tested, batch (a) was the one giving the lower values for activity and batch (b) was the one giving the highest activity.

abolished by saturating concentrations of EF 2 (figs. 1–3), but can be enhanced by increasing the concentration of free ribosomes (table 1). Indeed free ribosomes, even in the absence of fusidic acid, partially inhibit translocation (table 1) probably by sequestering also EF 2.

Fusidic acid has been reported to inhibit both aminoacyl-tRNA binding (table 2 and [16–19]) and translocation (fig. 3, table 1 and [1–7]) under certain experimental conditions but the mode of action of fusidic acid remains unresolved. Certainly binding of aminoacyl-tRNA does not take place when the complex GDP–ribosome–EF 2–fusidic acid is formed (table 2 and [25]). However, we believe that experimental conditions used in table 2 might be somehow artificial since it would appear more physiological that binding of aminoacyl-tRNA to the ribosome would take place prior to EF 2 interaction. Otherwise it would be difficult to understand how elongation could take place if EF 2 + GTP is bound to the ribosome prior to aminoacyl-tRNA binding since this binding is strongly inhibited by the previous interaction of EF 2 + GTP even in the absence of fusidic acid (table 2).

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