LONG TERM EFFECTS OF FUSIDIC ACID ON BACTERIAL PROTEIN SYNTHESIS IN VIVO.

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SUMMARY

Ribosomes continue to function, very slowly, in the presence of high concentrations of fusidic acid in vivo. Initially, the drug confines nascent peptides to the ribosomal P sites by inhibiting the binding of aminoacyl-tRNA into the A sites. Subsequently, the nascent peptides gradually escape from the P sites and a slow cycle is established in which the peptides enter and leave both ribosomal sites. At this stage fusidic acid also inhibits translocation. We have attempted to rationalize these findings in terms of interaction between fusidic acid, guanine nucleotides and elongation factors at a single ribosomal locus.

INTRODUCTION

Fusidic acid inhibits bacterial protein synthesis by interacting with elongation factor G (EF G) and inhibiting the dissociation of [ribosome-G-GDP] complexes formed during translocation (1). This gave rise to the idea that the drug inhibits the translocation reaction. However, according to a more recent hypothesis (2,3) bacterial ribosomes possess a single "GTPase" site, located in or near the 50S moiety of the A site, which is utilised alternately by elongation factors G (during translocation) and Tu (during the binding of aminoacyl-tRNA into the A site). This model predicts that EF G and EF Tu cannot interact simultaneously with a given ribosome. In agreement with this prediction, it was recently shown (4) that the primary consequence of the action of fusidic acid in vivo (in intact bacterial protoplasts) was to prevent the binding of aminoacyl-tRNA into the ribosomal A site under conditions where at least one round of translocation still occurred. This finding suggests that ribosomes bearing EF G and GDP in the presence of fusidic acid cannot bind aminoacyl-tRNA from its complex with EF Tu and GTP; such an effect has been demonstrated in vitro (5-8).

Ribosomes, G factor, GDP and fusidic acid form complexes which are not covalently bonded and which are not permanently stable *in vitro* (half-life around 5 min. at 0^{o}) even in the presence of quite high concentrations (3mM) of the drug. Here we have examined the stability of such complexes *in vivo* at 37^{o} by following the long-term effects of fusidic acid upon protein synthesis in intact protoplasts. We find that a proportion of the nascent peptides which are initially restricted to the ribosomal P sites by fusidic acid eventually escape from those sites and appear to be blocked in the A sites. This implies that fusidic acid can *secondarily* inhibit translocation. We have attempted to rationalize these observations with those previously reported from this and other laboratories.

METHODS

These have been discussed fully elsewhere (3,9,10). Protoplasts of *Bacillus megaterium* KM were prepared from cells containing ribosomes labelled with ^{32}P-phosphate for 3 generations and nascent peptides were labelled by incubating protoplasts with ^{3}H-leucine for 30 sec. at 37^{o} prior to the addition of various antibiotics as in the text. Lysates were prepared and analysed by sucrose density-gradient centrifugation. One refinement of the earlier methods was adopted; the lysis medium and the sucrose gradients contained chloramphenicol (100 µg/ml final concentration). Since this drug inhibits peptidyl transferase activity, its presence ensured that puromycin reactions could not occur during the analysis of lysates.

RESULTS AND DISCUSSION

As reported previously (4), when fusidic acid (300 µg/ml final concentration) was added to intact bacterial protoplasts, almost all the nascent peptides were confined initially (i.e. during the first 2-3 min. of incubation with the drug) to the P sites of ribosomes so that they reacted readily with puromycin (see also Table 1). Hence, fusidic acid *primarily* inhibits the binding of aminoacyl-tRNA into the A sites of ribosomes (4). However, we now report that following prolonged incubation (i.e. 20-30 min.)

TABLE 1

VARIATION IN THE PROPORTION OF NASCENT PEPTIDES RELEASABLE BY PUROMYCIN DURING PROLONGED INCUBATION OF PROTOPLASTS WITH FUSIDIC ACID.

DURATION OF TREATMENT WITH FUSIDIC ACID	NASCENT PEPTIDES ON RIBOSOMES AFTER INCUBATION WITH PUROMYCIN (% of those present when puromycin added)
20 sec.	13
2 min.	18
5 min.	23
10 min.	34
20 min.	37
30 min.	36

VARIATION IN THE PROPORTION OF NASCENT PEPTIDES RELEASABLE BY PUROMYCIN DURING PROLONGED INCUBATION OF PROTOPLASTS WITH FUSIDIC ACID.

Protoplasts, steady-state labelled with ^{32}P-phosphate, were incubated for 30 sec. at 37° with ^{3}H-leucine before fusidic acid (300 µg/ml final concentration) was added. Incubation was continued at 37° and, at the times indicated, portions of the suspension were transferred to flasks containing puromycin (25 µg/ml final concentration) and incubation was continued at 37° for a further 60 sec. During the incubations with puromycin, control samples (not treated with puromycin) were taken from the parent suspension. Samples were taken into DNase, RNase and Triton X100 and the resultant lysates were instantly frozen in acetone/CO_2. After thawing at 0°, lysates were analysed on sucrose density-gradients as previously described (3,9). Fractions from the gradients were precipitated with trichloroacetic acid, filtered on glass fibre discs and their radioactivity estimated as previously described (3,9).

Nascent peptides were estimated as ^{3}H in polyribosomes/total ^{32}P in ribosomes, i.e. as ^{3}H at 70S/ ^{32}P at 70S + 50S + 30S (see 9,10).

of protoplasts with fusidic acid, about 30-35% of the nascent peptides were found to be resistant to release by puromycin (Table 1). In discussing these results we have assumed, in accordance with our own work (10) and that of others (11,12), that fusidic acid does not, under any circumstances, affect

the peptidyl transferase reaction and that those nascent peptides which fail to react with puromycin here must, ipso facto, be located in the ribosomal A site. Since (Table 1) about one-third of the nascent peptides failed to react with puromycin (i.e. failed to enter the P sites of ribosomes) during 60 sec. of incubation following prolonged treatment of protoplasts with fusidic acid, it is clear that fusidic acid can inhibit translocation under such circumstances. Again we stress that the drug does not inhibit translocation immediately upon addition to protoplasts but only after prolonged incubation in vivo.

The final inhibited state of ribosomes in the presence of fusidic acid could, in principle, be either static or dynamic. A static state would be one in which the nascent peptides were distributed approximately 2:1 between the P and A sites and were immobilized; a dynamic state would involve movement of peptides from one site to the other so that the steady-state distribution between the sites would be as above. To distinguish between these possibilities we used antibiotics of known modes of action.

Chlortetracycline (CTC) inhibits the binding of aminoacyl-tRNA into the ribosomal A site whereas erythromycin (ERY) inhibits the translocation reaction (for a review of the modes of action of these and other antibiotics see 13). We reasoned that if the final inhibited state of ribosomes in the presence of fusidic acid were static, then puromycin reactions occurring under such conditions ought not to be affected by the addition of other antibiotics excepting those which inhibit peptidyl transferase. If, however, the final inhibited state were a dynamic equilibrium, in which nascent peptides were changing sites albeit slowly, then CTC and ERY (which do not affect peptidyl transferase) ought to be capable of trapping nascent peptides and preventing them from cycling between the sites. Thus, CTC should prevent the P $\longrightarrow$ A site transition by preventing aminoacyl-tRNA from binding into the A site and acting as an acceptor of the nascent peptide in the peptidyl transferase reaction. According to this scheme, CTC should cause a

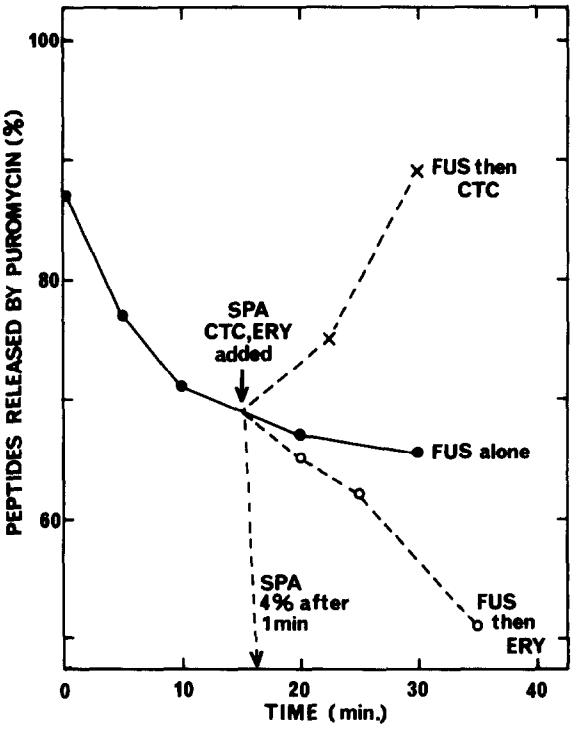

Fig. 1.

EFFECTS OF CHLORTETRACYCLINE AND ERYTHROMYCIN ON THE PUROMYCIN REACTION IN VIVO FOLLOWING PROLONGED TREATMENT WITH FUSIDIC ACID.

Experiment carried out as in Table 1. After 15 min. incubation with fusidic acid, portions of the protoplast suspension were transferred to flasks containing either CTC (200 μg/ml final concentration), ERY (300 μg/ml final concentration) or SPA (200 μg/ml final concentration). Incubations were continued at 37° and at the times indicated aliquots were taken into flasks containing puromycin for 60 sec. as in Table 1. Again, control samples (not treated with puromycin) were taken. Lysates were prepared and analysed as in Table 1.

Abbreviations: CTC (chlortetracycline), ERY (erythromycin), SPA (sparsomycin), FUS (fusidic acid)

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progressive accumulation of nascent peptides in the ribosomal P sites (i.e. free to react with puromycin) when added to protoplasts previously incubated extensively with fusidic acid. Conversely, ERY should cause nascent peptides to accumulate in the A sites of ribosomes under such conditions by preventing their translocation into the P sites.

The data given in Figure 1 indicate that the final inhibited state of

ribosomes in the presence of fusidic acid in vivo is a dynamic state. Following incubation of protoplasts with fusidic acid for 15 min., the proportion of nascent peptides released by puromycin treatment gradually changed from about 70% to around 90% during a further 15 min. of incubation in the presence of CTC. Under similar circumstances the proportion of puromycin-sensitive peptides eventually decreased to 50% when ERY was used instead of CTC. Figure 1 also shows that, as expected, inhibitors of peptidyl transferase (e.g. sparsomycin) almost completely inhibited puromycin reactions in this system immediately upon addition.

A further demonstration that nascent peptides continue to shift from one ribosomal site to the other in the presence of fusidic acid is given in Figure 2. In this experiment CTC or ERY was added to protoplasts a few seconds after the addition of fusidic acid. Under these conditions, CTC prevented the process whereby some of the nascent peptides would have become insensitive to the action of puromycin (compare control with fusidic acid alone), whereas ERY increased the proportion of non-releasable peptides. These effects are readily rationalized in terms of the inhibition of a slow, cyclic process as discussed above.

We therefore conclude that, immediately following the addition of fusidic acid to intact protoplasts, virtually all the nascent peptides are confined to the ribosomal P sites. Presumably this occurs according to the mechanism proposed by Bodley and co-workers (1) and involves the stabilization of [ribosome-G-GDP] complexes on the ribosomal GTPase site. Subsequently, the fusidic acid-stabilized complexes might dissociate thereby freeing the GTPase sites and allowing binding of [aminoacyl-tRNA-Tu-GTP] complexes into the now-accessible A sites. Once bound, aminoacyl-tRNA molecules could accept nascent peptides from the P sites. Apparently, in the presence of fusidic acid, peptides can be held in the A sites although not as effectively as in the P sites.

It may be that all these effects can be explained solely in terms of the

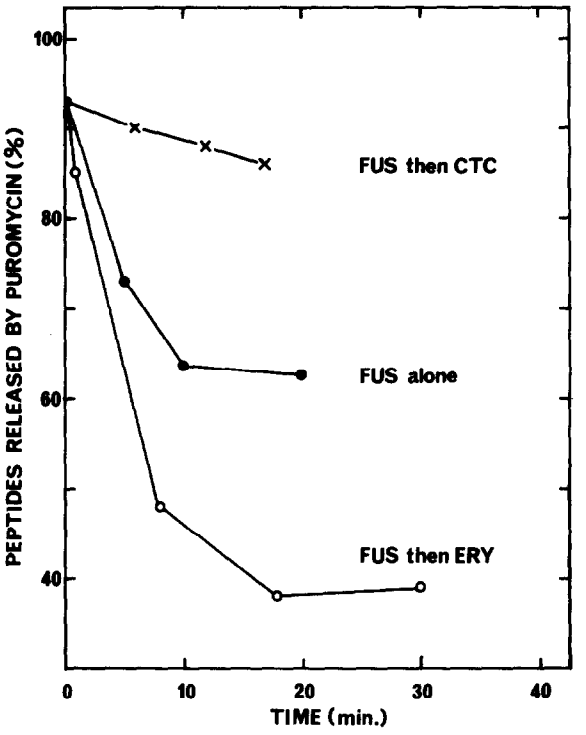

Fig. 2.

EFFECTS OF CHLORTETRACYCLINE AND ERYTHROMYCIN ON THE PUROMYCIN REACTION IN VIVO WHEN ADDED IMMEDIATELY AFTER FUSIDIC ACID.

This experiment was similar to that described in Figure 1 except that here treatment with CTC or ERY commenced 20 sec. after the addition of fusidic acid to protoplasts.

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stabilization of [ribosome-G-GDP] complexes by fusidic acid. Even in the presence of such complexes, aminoacyl-tRNA might be able to bind, with poor efficiency, to a proportion of the ribosomes. This is suggested by in vitro studies (5,6,8) where ribosomes in the presence of EF G, fusidic acid and GDP still bound about 30% of the control amounts of aminoacyl-tRNA. It is far from clear what the final state of the ribosomes would be under such circumstances since it is not obvious how EF Tu could bind to a ribosome already carrying EF G. However, the continued presence of EF G and GDP on such a ribosome might inhibit translocation by excluding GTP.

An alternative and intriguing possibility is that fusidic acid might stabilize ribosome-based complexes containing GDP and EF Tu which might be less stable than the corresponding complexes containing EF G. According to this hypothesis, which suggests that EF G and EF Tu might derive from a common ancestor, such putative [ribosome-Tu-GDP] complexes would block the ribosomal GTPase site in the presence of fusidic acid and would inhibit translocation by preventing the access of EF G to that site. Using Bodley's techniques (14), involving sucrose gradient centrifugation or "Sepharose" gel filtration, we have been unable to detect any such complexes involving EF Tu although those containing EF G were readily detected. In these experiments (not detailed here) our substrates were 70S ribosomes and either Tu-GTP or Tu-GDP: it may be that [aminoacyl-tRNA-Tu-GTP] complexes are required as starting material assuming this hypothesis is valid.

Finally, we are aware that fusidic acid-treated protoplasts offer a useful diagnostic system in which the modes of action of other antibiotics might be investigated *in vivo*. In particular this system, in which ribosomes function extremely slowly, might be useful for studying those antibiotics whose action is not efficient enough to allow them to be studied in otherwise uninhibited protoplasts (10).

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