TEMPERATURE-DEPENDENT INHIBITION OF POLYPHENYLALANINE FORMATION BY THE EXOTOXIN OF *BACILLUS THURINGIENSIS*

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1. Introduction

RNA polymerase from bacteria, insects and mammals is inhibited by the exotoxin produced by *B. thuringiensis* [1–3]. Observations in vitro and in vivo are in agreement with RNA polymerase being the site of attack in whole organisms and it has been suggested that the exotoxin acts as an analogue of ATP [4,5]. However, some in vivo observations [6] seem to suggest that protein synthesis as well as RNA synthesis may be affected. The present investigation demonstrates that at high concentrations exotoxin inhibits cell-free protein synthesis and that this inhibition is temperature sensitive.

2. Materials and methods

2.1. *Micro-organism*

E. coli MRE 600 was kindly donated by Dr M. Wilde, University of Oxford. Cells were harvested during exponential growth on Oxoid nutrient broth supplemented with 0.5% glucose. Cell-free extracts (S30, 30 000 *g* 30 min supernatant) were prepared by a modification, described previously [7], of the procedure of Wilhelm and Haselkorn [8].

2.2. *Assay for [^{14}C]phe-incorporation into polyphenylalanine*

The incubation mixture, based on that of Nirenberg [9], contained the following: 80 μl Mix II (see below); 2 μg poly U; S30, 2 mg protein in 100 μl of 0.01 M Tris–HCl + 0.01 M magnesium acetate, pH 7.6; 50 μg t-RNA; and distilled water to 250 μl. Mix II contained: 250 μl Mix I (see below); 1 μl 2-mercaptoethanol; 250 μl 0.075 M phosphoenolpyruvate, potassium salt; 4 μl phosphoenolpyruvate kinase (10 mg/ml, Sigma Chemical Co.); 50 μl 0.01 M [^{14}C]phenylalanine (4 μCi/μmole, Radiochemical Centre, Amersham, Bucks, U.K.) Mix I contained: 10.0 ml 2 M Tris–HCl, pH 7.8; 2.0 ml 1.4 M Mg-acetate; 5.0 ml 2 M KCl; 3.0 ml 0.066 M ATP, sodium salt; 3.0 ml 0.002 M GTP, sodium salt. Incubations were carried out at 37°C, except where indicated and were started by the addition of S30. For each determination of [^{14}C]phenylalanine incorporation, samples were taken and rapidly syringed onto 25 mm diameter Whatman 3MM paper discs which were dropped into precooled 5% trichloroacetic acid (TCA) containing a trace of unlabelled phenylalanine. The filters were transferred to boiling 5% TCA for 10 min then washed sequentially in the following [10]: cold 5% TCA for 30 min (twice); absolute ethanol for 10 min and diethyl ether for 10 min. The filters were finally dried under an infrared lamp and counted in 0.7% butyl-PBD [2-(4′-*t*-butylphenyl)-5-(4′-biphenyl)-1,3,4-oxadizole] (Intertechnique Limited, Portslade, Sussex) in toluene.

Where it was desired to estimate both incorporation into both aminoacyl-tRNA and into polypeptide, the following procedure was adopted. Filters were washed as described above but omitting the treatment in hot TCA. After counting, each filter was removed from the scintillation vial, washed twice for a few seconds in toluene, and blotted dry. The filters were then rewashed, including a hot TCA extraction; and recounted. Control experiments showed that there was no difference between polypeptide formation measured in this way and that measured by direct extraction with hot TCA.

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In a few experiments the MS2 RNA-mediated system was used as described previously [7].

2.3. *[^{14}C]phenylalanyl-tRNA*

[^{14}C]phenyl-alanyl-tRNA was prepared by the procedure of Nishizuka et al. [11] using the 150 000 *g* supernatant of an *E. coli* extract prepared according to Ravel and Shorey [12]. Exotoxin from *B. thuringiensis* was kindly donated by Dr R. P. M. Bond. Deaminated exotoxin was prepared according to Sebesta and Horska [13]. Poly-U and poly-A were obtained from Sigma (London) Chemical Co. Transfer RNA was obtained from Bohringer Corp.

3. Results

3.1. *Inhibition of polyphenylalanine formation by exotoxin*

Initial experiments indicated that, at relatively high concentrations, exotoxin inhibited MS2-mediated cell-free protein synthesis. Accordingly, the effect on the poly-U-mediated system was investigated and marked inhibition was observed (fig.1), again at high concentrations. Assay under conditions where incorporation into the cold-TCA-insoluble fraction (phe-tRNA plus polyphe) and the hot-TCA-insoluble fraction (polyphe only) was sequentially measured on the same samples indicated that the inhibition occurred after formation of the charged tRNA (fig.2).

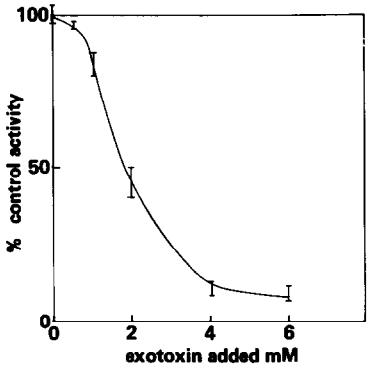

Fig.1. Effect of exotoxin on polyphenylalanine formation. The reaction mixture contained in 120 μl: Mix II, 40 μl; tRNA, 50 μg; poly-U, 3 μg; S-30, 25 μl and exotoxin as indicated.

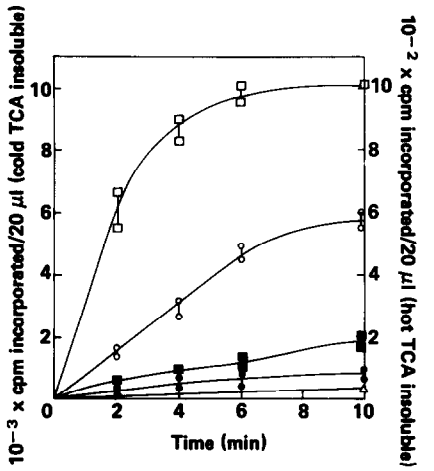

Fig.2. Effect of exotoxin on [^{14}C]phenylalanine incorporation into phe-tRNA and polyphenylalanine. The reaction mixture contained in 260 μl; Mix II, 80 μl; tRNA, 100 μg; poly-U, 3 μg; preincubated S-30, 10 μl (250 mg protein). At the times indicated 20 μl aliquots were removed and extracted with cold TCA as described in the Methods. After counting, the filters were reextracted with hot TCA and counted again. (□) cold-TCA-extracted. Determinations for the complete system, complete + exotoxin (4 mM), complete + deaminated exotoxin (2 mM) and complete - poly-U acid were within range indicated. (○) Hot-TCA-extracted, complete. (●) Hot-TCA-extracted, complete + 4 mM exotoxin. (■) Hot-TCA-extracted, complete + 2 mM deaminated exotoxin. (▲) Hot-TCA-extracted, complete-poly-U.

This was confirmed by measuring incorporation into polyphe from [^{14}C]phe-tRNA: the inhibition pattern was closely similar to that in fig.1. The effect was not confined to the poly-U-mediated system; polyadenylic acid-mediated [^{14}C]lysine incorporation was also inhibited.

3.2. *Effect of temperature on inhibition of polypeptide formation by exotoxin*

Some inconsistencies in the inhibition were observed from experiment to experiment and these were eventually traced to the use of a non-circulating water bath for temperature control. When experiments were carried out at accurately controlled temperatures the inhibition showed a marked dependence on temperature (fig.3). For example 4 mM exotoxin exerts no inhibitory effect at 25°C but causes 30% inhibition at 34°C and 70% at 39°C.

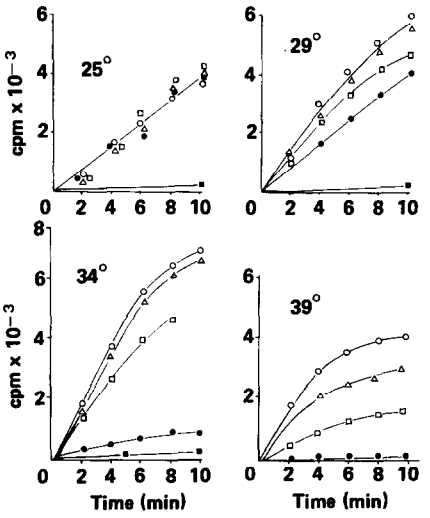

Fig.3. Temperature dependence of inhibition of polyphenylalanine formation by exotoxin. Incubation mixtures as in fig.1 but 250 μl total volume. Radioactivity was determined in duplicate 20 μl aliquots at the times indicated. (○) No exotoxin. (△) 2 mM exotoxin. (□) 4 mM exotoxin. (●) 8 mM exotoxin. (■) No Poly-U.

3.3. *Effect of Mg^{2+} and ATP on inhibition by exotoxin*

As exotoxin is a structural analogue of ATP the effect of ATP was compared with that of exotoxin. Whereas the concentration dependence of inhibition by sodium ATP was closely similar to that of exotoxin at 37°C, magnesium ATP had relatively little effect (20% inhibition at 6 mM). The effect of exotoxin was the same whether the exotoxin was added as the ammonium or sodium salt. Although Mg^{2+} is inhibitory at high concentrations (50% inhibition at 14 mM added Mg^{2+}) 5 mM Mg^{2+}, which was not inhibitory, did not relieve inhibition by sodium ATP. Similarly magnesium ATP did not affect the inhibition by exotoxin (table 1).

The inhibition of polyphenylalanine formation by sodium ATP differed from that by exotoxin in that it was independent of temperature over the range examined (24°–39°C).

4. Discussion

The exotoxin of *B. thuringiensis* is known to inhibit mammalian and insect RNA-polymerases at concentrations orders of magnitude less than those shown to inhibit polypeptide formation in the present work in which at least 2 mM exotoxin was required to inhibit cell-free protein synthesis. Although caution should obviously be exerted in extrapolating observations on microbial polypeptide formation to insect or mammalian systems, it seems unlikely that inhibition of protein synthesis plays a significant role in the in vivo effects of this toxin. The evidence clearly indicates an effect on translation rather than transcription.

The inhibition of protein synthesis at high concentrations appears to be a common property of nucleo-

Table 1
Effect of magnesium ATP on exotoxin inhibition of polyphenylalanine formation

Additions to reaction mixture		cpm incorporated/20 μl	% control
Mg^{++} ATP (mM)	Exotoxin (mM)		
0	0	2600	100
1.3	0	2840	110
5.2	0	2580	99
0	2	1500	58
1.3	2	1750	66
5.2	2	1740	66
0	4	644	22
1.3	4	798	27
5.2	4	703	24

Incubations were carried out at 37°C for 8 min.

tides as it has been previously reported that ATP inhibits protein synthesis [14] in mammalian cell-free systems. However, the inhibition was reported to be fully reversible by added Mg^{2+}.

Potentiometric studies [15] have shown that bivalent metal ions form complexes with nucleotide phosphates and that the stepwise deletion of phosphoryl groups from a nucleotide triphosphate causes a progressive decrease in the stability of the metal-nucleotide complex. In the present work observations with other nucleotides (for example 5.5 mM sodium GTP, GDP and GMP caused 100%, 74% and 46% inhibition respectively) may reflect the stability of the metal nucleotide complex. This would also explain the temperature dependence of inhibition by exotoxin: at room temperature the complexing of magnesium with exotoxin shows a temperature dependence remarkably similar to that of the inhibition described here for protein synthesis, (R. P. M. Bond and the late D. Eaton, A. K. Handa, personal communication). The binding of Mg^{2+} may not be the only factor in the inhibition as ethylenediamine diacetic acid, which has a similar affinity for Mg^{2+}, did not have any inhibitory effect at equivalent concentrations. That the inhibition is not specific for analogues of ATP was suggested by a single experiment at 37°C with deaminated exotoxin (GTP analogue which inhibits incorporation from GTP by RNA polymerase) in which similar inhibition was observed.

It would appear that for inhibition both a structural relationship to a nucleotide and the ability to bind magnesium are necessary. The temperature dependence of the inhibition by exotoxin may be useful in studies of nucleotide-ribosome interactions.

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