INHIBITION AT THE INITIATION LEVEL OF EUKARYOTIC PROTEIN SYNTHESIS BY T-2 TOXIN

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1. Introduction

The 12,13-epoxytrichothecenes inhibit protein synthesis in eukaryotic organisms [1] and can block peptidyl transferase activity as assayed by the puromycin fragment reaction [2]. However, on the basis of experiments in vivo they have been divided into two subgroups [3]. It has been proposed that compounds in one subgroup (I-type inhibitors) which include T-2 toxin, act at some stage of the initiation process. Compounds in the second subgroup (E-type inhibitors) include trichodermin and seem to inhibit elongation or perhaps more specifically termination [4].

The 12,13-epoxytrichothecenes are closely related chemically [5] and it has been suggested that they have the same site of action on ribosomes [6]. It remains, therefore, to establish why the two subgroups apparently act at different stages of the protein synthetic cycle. Recent work with trichodermin has strongly suggested that this compound acts as a general inhibitor of the peptidyl transferase catalytic centre [6] and it is tempting to assume that T-2 toxin also inhibits this same enzymic centre but, for some reason, only at the initiation step of protein synthesis.

In this present work we have utilized the reticulocyte cell-free system to study the effect of T-2 toxin on the initiation process. We have found that T-2 toxin can inhibit the reaction which can take place between puromycin and met-tRNA$_f^{met}$ bound to the 80 S ribosome.

2. Materials and methods

2.1. *Preparation of rabbit reticulocyte lysates*

Blood from anaemic young male New Zealand white rabbits was collected in ice-cold 100 ml glass centrifuge tubes. The reticulocytes were harvested from the blood and then washed three times in 130 mM NaCl–5 mM KCl–7.5 mM magnesium acetate before resuspension (original volume of the packed cells) in 4 mM magnesium acetate–1 mM dithiothreitol. After gentle stirring for 5 min unlysed cells and debris were removed by centrifugation for 20 min at 12 000 rev/min in a M.S.E. 18 centrifuge. The supernate (1:1 lysate) was decanted and stored in 1 ml aliquots in liquid nitrogen.

2.2. *Cell-free protein synthesis in reticulocyte lysates*

Cell-free protein synthesis was assayed by adding 125 μl of 1:1 lysate to an equal volume of incubation mix (pH 7.2) so that the final concentrations of materials were 80 mM KCl, 20 mM Tris–Cl buffer, pH 7.2 at 20°C, 3 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM EDTA, 20 mM creatine phosphate, 1 mM ATP, 1.6 mM GTP, 2×10^{-2} mM of unlabelled amino acids without valine, 4 μCi of L(2,3-[^{3}H]) valine-specific activity 31.6 Ci/mmol and 5 μg of creatine kinase.

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The mixture was incubated at 36°C and at appropriate times 20 μl aliquots were withdrawn, pipetted on to 2.5 cm discs of Whatman 3 mm paper and placed in a beaker of ice-cold acetone containing 1% (v/v) HCl to precipitate protein and remove haem. 30 min after the addition of the last disc to the beaker, the discs were removed and placed into 5% trichloroacetic acid preheated to 95°C. After 20 min at 95°C the discs were removed and washed twice with each of ice-cold 5% trichloroacetic acid, ethanol–ether (3:1 v/v) and ether. After drying, the discs were counted in a liquid scintillation counter.

2.3. *Preparation of 0.5 M KCl ribosomal wash*

Reticulocyte 0.5 M KCl ribosomal wash was prepared from total reticulocyte ribosome pellets as described by Schreier and Staehelin [7].

2.4. *Preparation of 0.5 M KCl derived ribosomal subunits from Krebs ascites tumour cells*

Krebs ascites cells were grown for seven to eight days in male Schofield mice. The cells were harvested and ribosomal subunits were prepared by sucrose gradient centrifugation of cell homogenates as described previously [8].

2.5. *Preparation and analysis of [^{35}S] methionyl-tRNA$^{met}_f$*

Partially purified preparations of *Escherichia coli* aminoacyl-tRNA synthetases were used to charge stripped rabbit liver tRNA with L-[^{35}S] methionine, specific activity 190 Ci/mmole, as previously described [9]. Analysis of the charged tRNA preparations has previously shown [9] that it consists almost exclusively of met-tRNA$^{met}_f$.

2.6. *Formation of 80 S ribosome initiation complex*

0.39 A_{260} units of 0.5 M KCl washed 40 S and 0.62 A_{260} units of 60 S ribosomal subunits derived from Krebs ascites cells were incubated in 1 ml of a solution containing as final concentrations 80 mM KCl, 35 mM Tris–Cl buffer, pH 7.2 at 20°C, 3 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM EDTA, 10 μg of a 1:1:1 random copolymer of poly (A,U,G), 0.1 mM GTP, 2.5×10^5 cpm of L-[^{35}S] met-tRNA$^{met}_f$ and 40 μl of reticulocyte 0.5 M KCl ribosomal wash. After 10 min incubation at 36°C, the mixtures were cooled to 0°C and 0.65 ml was layered on to a 4.5 ml linear 10–30% sucrose gradient made up in the incubation buffer. The gradients were centrifuged for 45 min at 50 000 rev/min and 2°C in a Spinco S.W. 50.1 rotor. The gradients were eluted by piercing a hole in the bottom of the tubes and 11 drop (350 μl) fractions were collected. 200 μl of each fraction was pipetted on to 2.5 cm Whatman GF/C filter discs in successive 50 μl aliquots. The filters were dried thoroughly before counting in a liquid scintillation counter.

T-2 toxin and cycloheximide were dissolved in dimethylsulphoxide and water respectively. Final concentrations of dimethylsulphoxide in experimental reaction mixtures were always less than 1% (v/v). In control experiments dimethylsulphoxide did not contribute to any of the effects reported here. Puromycin dihydrochloride was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, USA. Poly(A,U,G) was purchased from P.L. Biochemicals, Milwaukee, Wisconsin, USA. All radioactive chemicals were purchased from the Radiochemical Centre (Amersham, England).

3. Results and discussion

In HeLa cells and in yeast spheroplasts addition of T-2 toxin caused rapid and almost complete breakdown of polyribosomes [3]. This was the main indication that T-2 toxin acts at the level of initiation since the effect was paralleled by a rapid inhibition of protein synthesis which became apparent shortly after addition of the drug. Here we have used the reticulocyte cell-free system to study the effect of T-2 toxin on protein synthesis, in vitro, since this system has been relatively well-characterized and thus provides a means to examine some of the individual steps involved in the initiation process [10]. Prior to the present work, there had been no detailed investigation of the effects of trichothecenes upon polypeptide chain initiation nor indeed was there any evidence that any of these compounds selectively inhibit chain initiation in vitro.

In fig.1 the effect of adding different trichothecenes to a reticulocyte lysate synthesizing protein is illustrated. Uptake of valine into protein normally proceeds linearly for approximately 5 min before the curve tails off. Addition, after 2 min incubation, of T-2 toxin (80 μg/ml) has no effect on protein synthesis for about

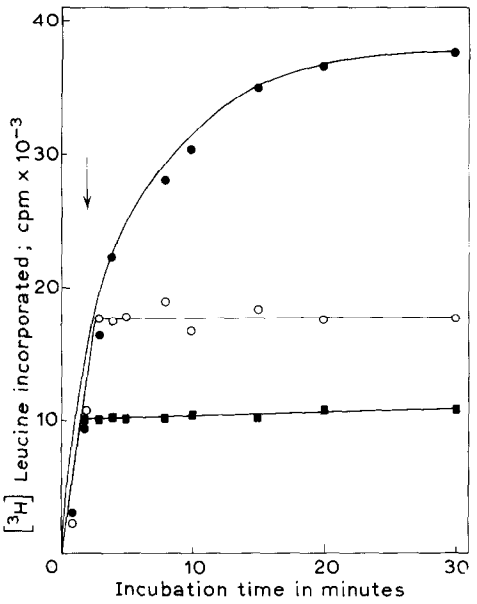

Fig.1. The effect of T-2 toxin on protein synthesis in reticulocyte lysates. The assay mixture, prepared as described in materials and methods, was incubated at 36°C. After 2 min (indicated by the arrow in the diagram) the inhibitors, dissolved in dimethylsulphoxide for T-2 toxin and water for cycloheximide, were added to the incubation. An equivalent volume of dimethylsulphoxide was added to the control. Aliquots of the reaction mixtures were removed at the times indicated and processed and counted as described in materials and methods. (●–●–●) control; (o–o–o) T-2 toxin; (■–■–■) cycloheximide (1.6 mg/ml final concentration), with trichodermin (40 μg/ml final concentration) identical points were obtained.

60 sec but then protein synthesis is inhibited abruptly. This lag is absolutely reproducible and is significant. Such kinetics of inhibition are consistent with the idea that T-2 toxin (an I-type inhibitor in vivo) inhibits the initiation of protein synthesis and allows those ribosomes already synthesizing protein at the time of drug addition to complete their translation cycles. In contrast, trichodermin (an E-type inhibitor in vivo) inhibited similar systems promptly, with no suggestion of a lag, in keeping with the notion that this drug blocks polypeptide chain elongation. Inhibition by cycloheximide, a known inhibitor of peptide chain elongation, follows identical kinetics to those shown by trichodermin.

The foregoing results establish, for the first time, that the trichothecene antibiotics inhibit protein synthesis in vitro in a manner analogous to that observed in vivo and they support the division of these compounds into distinct functional types [3]. Chemically, however, the compounds are very similar, and whether or not the nature of substituents on carbon-15 is responsible for determining their precise modes of action [3], it seems likely that the various compounds within this group will bind to the same ribosomal site(s). In other hands [2] a number of trichothecenes, comprising both E-types and I-types, behaved similarly in inhibiting puromycin-dependent release of *N*-acetyl leucine from aminoacyl-oligonucleotide 'fragments' (ACCA–leuAc) of tRNA in the presence of human tonsil ribosomes. Furthermore [6], the release, by puromycin, of nascent peptides from polyribosomes derived from rat liver was inhibited by trichodermin (and other E-type inhibitors). We, therefore, considered it important to investigate the effects of T-2 toxin upon pyromycin reactions utilising

Table 1
Release of nascent chains from washed reticulocyte polyribosomes by puromycin

	% nascent chains released
a) Control – no incubation	0
b) Control incubated for 10 min at 37°C	5
c) Control incubated for 10 min at 37°C with T-2 toxin (40 μg/ml)	0
d) Control incubated for 10 min at 37°C with puromycin (1 mg/ml)	68
e) Control incubated for 1 min at 37°C with T-2 toxin (40 μg/ml) followed by a further 9 min incubation at 37°C with puromycin (1 mg/ml)	64

The nascent chains on reticulocyte polyribosomes were labelled by incubating 2 ml of reticulocyte lysate for 2 min at 37°C as described in section 2.2. After the incubation, the mixture was cooled to 0°C and layered over 6 ml of 20% sucrose made up in 20 mM Tris–Cl buffer, pH 7.2, 3 mM magnesium acetate, 1 mM dithiothreitol, 0.1 mM EDTA and 500 mM KCl. Washed polyribosomes were collected by centrifugation at 45 000 rev/min for 2 hr in a Spinco Ti50 rotor. The pellets obtained were rinsed twice with incubation buffer (section 2.2.) and resuspended in the same buffer to a final concentration of 22 A_{260} units/ml and 1.5×10^5 cpm/ml. 4.5×10^3 cpm of washed polyribosomes were incubated as described above.

reticulocyte polyribosomes and the results are given in table 1. Again, there was evidence to support a clear division of the trichothecenes into two groups; T-2 toxin failed to inhibit peptide release in this system whereas trichodermin, as shown previously, almost totally prevented release [6].

Because of the failure of T-2 toxin to inhibit peptide bond formation with polyribosomes we examined the effect of the drug on the initiation of protein synthesis. In reticulocyte cell-free protein synthesizing systems the initiation process involves several well-defined steps [10] which end with the formation of an 80 S ribosome to which met-tRNA$_f^{met}$ is bound in the P site. We have studied the effect of T-2 toxin on the reaction which can take place between puromycin and this bound met-tRNA$_f^{met}$, a reaction in many ways analogous to the first peptide bond formation in protein synthesis. In fig.2(b) we show that T-2 toxin does not prevent the formation of an 80 S initiation complex containing met-tRNA$_f^{met}$ (and also, by inference, mRNA) although such a complex formed in the presence of T-2 toxin does not react with puromycin (fig.2d). It is possible that an aberrant 80 S complex was formed in the presence of T-2 toxin. Alternatively T-2 toxin could inhibit the puromycin reaction per se (i.e. the peptidyl transferase reaction). In the control (fig.2c) incubation of 80 S initiation complexes with puromycin alone released approximately 75% of the ^{35}S-labelled methionine. To distinguish between the two possibilities outlined above an 80 S initiation complex was formed as described in fig.2(a). T-2 toxin and puromycin were then added simultaneously and the incubation continued for a further 5 min. The result was identical to that shown in fig.2(d). Thus, we conclude that T-2 toxin inhibits peptidyl transferase directly. In fig.2(c) there is a pronounced radioactive peak in the 40 S region of the gradient. This may represent complexes formed between ^{35}S-labelled met-tRNA$_f^{met}$ and 40 S ribosomal subunits generated during puromycin treatment. For some reason such complexes do not combine with 60 S subunits.

We deduce from the above experiments that T-2 toxin inhibits polypeptide chain initiation, not by preventing the assembly of the 80 S initiation complex but by preventing the formation of the first peptide bond in the protein synthetic cycle. Since trichodermin inhibits peptidyl transferase reactions in general [2,6] we consider that T-2 toxin probably acts on the same enzymic centre presumably by binding to the same ribosomal site(s) as trichodermin. Support for this supposition comes from the recent work of Schindler [6] who showed that verrucarin A

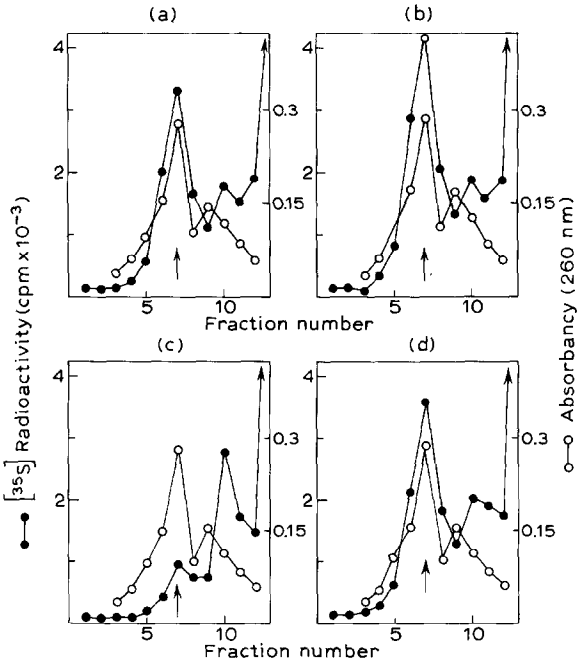

Fig. 2. The effect of T-2 toxin on the puromycin-induced release of methionine from tRNA$_f^{met}$ bound to 80 S ribosome complexes. The assay was performed as described in Materials and methods. a) Control 10 min incubation; b) T-2 toxin (40 μg/ml final concentration) added at zero time and the incubation was for 5 min; c) control samples were incubated for 5 min before addition of puromycin (1 mg/ml final concentration) and reincubation for a further 5 min; d) T-2 toxin (40 μg/ml) added at zero time and the mixture incubated for 5 min before addition of puromycin (1 mg/ml) and reincubation for a further 5 min. (●–●–●) ^{35}S-radioactivity; (○–○–○) superimposed position of an absorbancy profile (A_{260}) from an identical gradient to show sedimentation position of 80 S ribosome monomers. This gradient contained 0.3 A_{260} units of 40 S and 0.7 A_{260} units of 60 S ribosomal subunits. Under the salt conditions of the gradient 80 S monomers form and the excess 60 S subunits also appear on the absorbancy profile. For all the graphs the sedimentation is from right to left and the position of the 80 S monomer is indicated by the arrow. A further experiment was carried out in which T-2 toxin and puromycin were added simultaneously to a control complex formed over 5 min incubation. After a further 5 min incubation a result identical to the one shown in d) was obtained.

(an I-type inhibitor) and trichodermin share, at least in part, a common ribosomal binding site. Since T-2 toxin fails to inhibit peptidyl transferase reactions on reticulocyte polyribosomes (which possess nascent peptides of various lengths) we consider that our data is best explained according to the earlier hypothesis [3] whereby the presence (in the ribosomal P site) of oligopeptidyl-tRNA of an undefined but critical, minimum chain length can prevent the ribosomal binding of I-type trichothecenes. Apparently this binding is not prevented by the presence of met-tRNA$_f^{met}$ in the P site. The first peptide bond formation in protein synthesis need not, however, be a reaction uniquely inhibited by T-2 toxin and the second, third etc. peptide bond-forming steps may be sensitive to the drug.

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