Prevention, by Ribosome-Bound Nascent Polyphenylalanine Chains, of the Functional Interaction of T-2 Toxin with its Receptor Site

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1. The inhibitory effects of T-2 toxin and trichodermin on poly(U)-directed polyphenylalanine synthesis were studied by using cell-free systems from reticulocytes. Conditions for amino acid incorporation were carefully chosen in an attempt to ensure that the large majority of poly(U) chains bound only one ribosome engaged in protein synthesis and that all such ribosomes carried nascent polyphenylalanine chains containing approximately the same number of residues. 2. Cell-free systems were allowed to synthesize polyphenylalanine, and T-2 toxin and trichodermin were added to the incorporation mixtures at various times. Irrespective of the time of addition, trichodermin (50 µg/ml) inhibited polyphenylalanine synthesis by approx. 70%. In contrast, although T-2 toxin (40 µg/ml), when added at early incubation times, could inhibit polyphenylalanine synthesis with a maximum of 50%, the drug had no effect on the system when added after a critical time-period. 3. It is concluded that although both T-2 toxin and trichodermin can inhibit peptide-bond formation on ribosomes at the level of the peptidyl transferase catalytic centre the presence, on ribosomes, of nascent polyphenylalanine chains above a certain critical chain length excludes T-2 toxin from functional interaction with its receptor site.

The 12,13-epoxytrichothecenes inhibit protein synthesis in eukaryotic systems (Ueno, 1970; Bamberg & Strong, 1971), and, on the basis of their modes of action both *in vivo* and *in vitro*, they have been divided into two general groups (Cundliffe *et al.*, 1974). Compounds in one group, containing trichodermin and trichodermol, stabilize polyribosome profiles (Cundliffe *et al.*, 1974; Carter *et al.*, 1976), acting as inhibitors of either elongation or termination (Wei *et al.*, 1974), whereas those in a second group, containing T-2 toxin and verrucarins A, allow 'run off' of polyribosomes, consistent with an inhibitory effect at, or shortly after, the initiation step of protein synthesis.

The inhibitory actions of the various compounds have also been studied by using both the puromycin and the fragment reactions. These systems are well characterized and inhibition of them by a drug implies inhibition at the level of the peptidyl transferase centre on ribosomes. Both trichodermin and T-2 toxin inhibit the reaction between puromycin and methionyl-tRNA^{Met} bound to 80S ribosomal initiation complexes (Wei *et al.*, 1974; Smith *et al.*, 1975) and both trichodermin and verrucarins A are potent inhibitors of the fragment reaction (Carrasco *et al.*, 1973). However, although puromycin also reacts with nascent polypeptide chains bound through tRNA to the P sites of polyribosomes, it is significant that

although trichodermin strongly inhibits this particular reaction (Schindler, 1974; Smith *et al.*, 1975), T-2 toxin has no effect on the release of peptidyl-puromycin (Smith *et al.*, 1975).

From the above considerations it is difficult to decide whether T-2 toxin inhibits peptide-bond formation at some step unique to the initiation of protein synthesis or whether the drug is a general inhibitor of peptidyl transferase but is excluded functionally from this catalytic centre under certain conditions. In these investigations we have attempted to distinguish between these two possibilities.

Experimental

Preparation and preincubation of rabbit reticulocyte lysates for the poly(U)-dependent synthesis of polyphenylalanine

Blood from anaemic male New Zealand white rabbits was collected by cardiac puncture into ice-cold 100 ml glass centrifuge tubes. The reticulocytes were harvested by centrifugation at 1000g for 10 min in an MSE Magnum centrifuge, washed twice in 130 mM-NaCl/5 mM-KCl/7.5 mM-magnesium acetate, pH 7, and finally resuspended in an equal volume of water to lyse the cells. After 5 min of gentle stirring, unlysed cells and debris were removed by centrifugation at 17000g for 15 min in a Sorvall centrifuge.

A reticulocyte lysate containing 'run off' ribosomes was prepared by preincubating an equal volume of the fresh lysate with an incubation mixture to give a final concentration of 80 mM-KCl, 20 mM-Tris/HCl, pH 7.4, 3 mM-magnesium acetate, 1 mM-dithiothreitol, 0.1 mM-EDTA, 20 mM-creatine phosphate, 1 mM-ATP, 1.6 mM-GTP, 0.02 mM of each of 20 unlabelled amino acids and 20 μ g of creatine kinase (Boehringer, Mannheim, W. Germany)/ml. After incubation for 45 min at 37°C, the mixture was cooled to 0°C and exhaustively dialysed at 4°C for 16 h against several changes of water. Batches (3 ml) of preincubated lysate were stored at -70°C.

Poly(U)-dependent synthesis of polyphenylalanine in the preincubated lysate

The poly(U)-dependent synthesis of polyphenylalanine was assayed by adding 2.5 vol. of preincubated reticulocyte lysate to 1 vol. of incubation mixture (pH 7.4) to give a final concentration of 100 mM-KCl, 20 mM-Tris/HCl, 12 mM-magnesium acetate, 1 mM-dithiothreitol, 20 mM-creatine phosphate, 1.2 mM-ATP, 0.8 mM-GTP, 20 μ g of creatine kinase/ml and, unless otherwise stated, 200 μ g of poly(U)/ml. The mixtures were preincubated at 20°C for 10 min to allow the poly(U) to bind to the ribosomes. After the preincubation the synthesis of 14 C-labelled polyphenylalanine was started by adding 0.5 μ Ci of L-[14 C]phenylalanine (522 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) to 500 μ l of the assay mixtures. Samples (50 μ l) of incubation mixture were removed at appropriate times and pipetted on to Whatman 3MM paper discs, which were then placed into a beaker of ice-cold 1% (v/v) HCl in acetone to precipitate the polyphenylalanine and remove the haem. After 30 min the acid/acetone was removed and the discs were boiled for 20 min in 5% (w/v) trichloroacetic acid, washed twice in ice-cold 5% trichloroacetic acid and twice in ethanol before being dried and counted for radioactivity.

Sucrose-gradient analysis of the incubation mixtures

Cycloheximide (final concentration 1 mg/ml) was added to 50 μ l samples of incubation mixture, followed by 150 μ l of incubation buffer (80 mM-KCl, 20 mM-Tris/HCl, pH 7.4, 3 mM-magnesium acetate). The diluted incubation mixtures were then layered on to 4.8 ml linear sucrose gradients (10–30%, w/v) made up in incubation buffer. The gradients were centrifuged for 45 min at 40000 rev./min and 2°C in a Spinco SW50.1 rotor. To locate the position of the polyribosomes on the gradient, 20 μ l of freshly prepared lysate which had not been preincubated was diluted to 200 μ l with incubation buffer and placed on to a gradient which was run in parallel as a control. The absorbance profile of the gradients was obtained by upward displacement of the samples into an Isco u.v. flow analyser set to measure E_{254} ; six-

drop fractions were collected (volume 200 μ l) into separate tubes and then the whole of each fraction was pipetted on to 2.5 cm Whatman GF/C glass-fibre discs. The discs were dried and counted for radioactivity.

In all cases the samples containing radioactivity were counted in a Packard liquid-scintillation counter at an efficiency, for 14 C-labelled samples, of approx. 40%.

Materials

Trichodermin (mol.wt. 292) and T-2 toxin (mol.wt. 350) were kindly supplied by Mr. D. Schindler, Department of Biochemistry, University of Wisconsin, Madison, WI, U.S.A. Aurintricarboxylic acid and cycloheximide were both generously supplied by Dr. E. Cundliffe, Department of Molecular Pharmacology, University of Cambridge, Cambridge, U.K. All the drugs were dissolved in water, except for T-2 toxin, which was always dissolved in dimethyl sulphoxide. The concentration of dimethyl sulphoxide never exceeded 0.5% (v/v) when present in reaction mixtures.

Results and Discussion

The overall aim of our experiments was to study the inhibition by both T-2 toxin and trichodermin of poly(U)-directed polyphenylalanine synthesis in reticulocyte lysates. Our intention was to determine whether or not the length of the nascent polyphenylalanine chain on ribosomes affected the ability of these two drugs to inhibit protein synthesis.

Addition of poly(U) to a reticulocyte cell-free system stimulates phenylalanine incorporation and the amount of polyphenylalanine synthesized depends on the concentration of poly(U), amounts above 100 μ g/ml saturating the synthetic capacity of the system. The reticulocyte lysates used in the assay contained 'run-off' ribosomes, and for our experiments on drug inhibition we chose a poly(U) concentration of 200 μ g/ml. We hoped that synthesis of polyphenylalanine would start simultaneously on many poly(U) chains and that because ribosomes were limiting in the system relative to synthetic mRNA, no poly(U) chain would carry more than one ribosome. We also chose an incubation temperature of 20°C to slow down the rate of elongation. We intended to produce a protein-synthesizing system which was 'synchronized' as possible, with all nascent polyphenylalanine chains having the same number of residues and all elongating slowly although at the same rate.

A time-course of phenylalanine incorporation as directed by poly(U) (200 μ g/ml) was carried out. The incorporation proceeded in approximately linear fashion for 40 min (see Fig. 2 for control incorporation kinetics) and a sample was removed at 20 min

Fig. 1. Effect of poly(U) concentration on polyribosome size in run-down reticulocyte lysates

Incubation mixtures were prepared as described in the Experimental section by using either (○) 20 or (●) 200 μg of poly(U)/ml to direct phenylalanine incorporation. After 20 min incubation at 20°C, 50 μl samples were removed, mixed with cycloheximide and analysed on sucrose gradients for their labelled polyribosome content. Details are described in the Experimental section. An E_{254} profile of a freshly prepared reticulocyte lysate, prepared as described in the Experimental section, is illustrated as a control profile (—). The horizontal arrow under the abscissa represents the direction of sedimentation of polyribosomes in the gradient. The position of the 80S ribosome peak is marked with a vertical arrow.

for further analysis. A second incorporation was carried out under identical conditions except that the final poly(U) concentration was only 20 $\mu\text{g}/\text{ml}$. Kinetics of incorporation were similar to those above, and again a sample was removed after 20 min incubation. The samples were then analysed on sucrose gradients for their labelled polyribosome content, and the results are illustrated in Fig. 1. The sample removed from the incubation mixture containing the lower poly(U) concentration has a large labelled polyribosome content and 80S ribosomes represent approx. 30% of the label in the total preparation. In contrast, the other sample contains radioactive label almost entirely on 80S ribosomes and any labelled polyribosomes are restricted to a small shoulder in the region of a probable dimer. For comparison, Fig. 1 also illustrates a polyribosome absorbance profile from a reticulocyte lysate which has not been subjected to any preincubation treatment. The absorbance profile is similar to the labelled profile displayed by the sample from the cell-free system incubated with the lower poly(U) concentration. This result is encouraging in that saturating amounts of poly(U) clearly preclude the association, during protein synthesis under these conditions, of several ribosomes with each poly(U) chain and most chains appear to carry one polyphenylalanine-synthesizing ribosome only.

To substantiate these conclusions we then studied the effect of aurintricarboxylic acid on our system and the results are illustrated in Fig. 2. When aurintricarboxylic acid was added to run-down lysates just before the simultaneous addition of both poly(U) and ^{14}C -labelled phenylalanine, synthesis of polyphenylalanine was almost totally inhibited. This is consistent with the suggested mode of action of aurintricarboxylic acid. The drug inhibits protein synthesis at the initiation level by preventing the association of mRNA with ribosomal subunits (Grollman & Huang, 1970). In contrast, when the run-down lysate was mixed with poly(U) and preincubated for 10 min before simultaneous addition of both aurintricarboxylic acid and ^{14}C -labelled phenylalanine the drug had no effect on the subsequent synthesis of polyphenylalanine for at least a 20 min incubation period and showed only partial inhibition over the next 40 min incubation. This result suggests that most ribosomal subunits in the lysate have combined into 80S ribosomes bound to poly(U) during the preincubation period, and are therefore unaffected by aurintricarboxylic acid. The later inhibitory effect

Fig. 2. Effect of aurintricarboxylic acid on polyphenylalanine synthesis in run-down reticulocyte lysates

Incubation mixtures were prepared containing 200 μg of poly(U)/ml and incubated at 20°C. Samples (50 μl) were removed at the times indicated and processed for radioactivity as previously described. Control incorporation, ●; aurintricarboxylic acid (final concentration 50 μM) added to incorporation mixtures before addition of poly(U) and [^{14}C]phenylalanine, ■; aurintricarboxylic acid (final concentration 50 μM) added simultaneously with [^{14}C]phenylalanine to incorporation mixtures previously preincubated for 10 min with poly(U), □.

of the drug may be on that (small) proportion of the ribosome population which, for some reason, has failed to bind poly(U) in the initial preincubation stage. In the main, however, the above result is again consistent with a large degree of synchrony in the process whereby ribosomes bind to poly(U) and initiate protein synthesis under the experimental conditions used in this study.

Having characterized our system we next studied its inhibition by the two drugs T-2 toxin and trichodermin. Although both drugs are potent inhibitors of protein synthesis *in vivo* (Cundliffe *et al.*, 1974), the compounds only partially inhibit poly(U)-directed polyphenylalanine synthesis in reticulocyte lysates. Thus, as shown in the dose-response curves in Fig. 3, T-2 toxin never inhibits the system by more than 50%, although trichodermin inhibits with a maximum of 70%. We emphasize, however, that these responses are totally reproducible under the conditions of our experiments, although reports in the literature show variable inhibition by trichodermin of poly(U)-directed synthesis in extracts from different tissues. Thus cell-free systems from human tonsils and in particular from yeast are relatively insensitive to the drug when poly(U) is used to direct protein synthesis (Carrasco *et al.*, 1973; Schindler, 1974).

As outlined in the introduction, trichodermin stabilizes polyribosomes when added to a variety of systems both *in vivo* and *in vitro*, and such an effect is characteristic of an inhibitor of elongation. The drug

is thought to interact with the peptidyl transferase centre on ribosomes, since it is a potent inhibitor of both the puromycin reaction and the fragment reaction (Carrasco *et al.*, 1973). In particular, trichodermin inhibits the reaction of puromycin, not only with methionyl-tRNA^{Met} bound to the initiation complex (Wei *et al.*, 1974), but also with nascent polypeptide chains bound through tRNA to either mono- or poly-ribosomes (Schindler, 1974; M. Cannon, unpublished work). Trichodermin also inhibits the puromycin reaction with polyphenylalanine chains bound through tRNA to reticulocyte ribosomes (K. E. Smith, unpublished work).

In contrast with trichodermin, T-2 toxin induces 'run off' of polyribosomes when added *in vivo* (Cundliffe *et al.*, 1974), and we have extended these observations and found that the drug also induces 'run off' *in vitro* in reticulocyte lysates synthesizing protein (Carter *et al.*, 1976). These effects are consistent with inhibition at the initiation stage of protein synthesis or shortly thereafter, and, as with trichodermin, this could represent inhibition by T-2 toxin of peptidyl transferase (Carrasco *et al.*, 1973). Implicit in these observations is the possibility that T-2 toxin could inhibit the first peptide-bond-forming step in protein synthesis, because this particular peptidyl transferase reaction is uniquely different from all others in the protein-synthesizing cycle. This uniqueness could extend beyond the obvious consideration that at initiation the ribosomal P site is occupied by methionyl-tRNA^{Met}, as opposed to peptidyl-tRNA, which would occupy the P site at subsequent stages of elongation.

Although the above hypothesis is attractive it is relevant to consider the behaviour of T-2 toxin in assays of the puromycin reaction. The drug inhibits the reaction of puromycin with methionyl-tRNA^{Met} bound to the ribosomal initiation complex (Smith *et al.*, 1975). However, the drug fails to inhibit the puromycin reaction with nascent peptides bound to either rat liver polyribosomes or salt-washed yeast 80S ribosomes (Schindler, 1974). We have extended these observations and have shown that T-2 toxin also fails to inhibit the puromycin reaction with reticulocyte ribosomes (as either polyribosomes or 80S ribosomes) carrying nascent polyphenylalanine chains (results not shown). Although these results in themselves could support the suggestion outlined in the previous paragraph, they could also indicate that the presence of nascent polypeptide chains on ribosomes prevents the functional interaction of T-2 toxin with its receptor site(s). It will be clear from our above considerations that these same ribosomes can bind trichodermin and are strongly inhibited by the drug.

The position is clarified only slightly by studies on inhibition of the fragment reaction by a further 12,13-epoxytrichothecene, verrucaric acid (Carrasco *et al.*, 1973). This drug behaves similarly to T-2 toxin both

Fig. 3. Inhibition of polyphenylalanine synthesis in run-down reticulocyte lysates by T-2 toxin and trichodermin

Incorporation mixtures were prepared as described previously with addition of 200 µg of poly(U)/ml and incubated at 20°C for 30 min in the presence or absence of various concentrations of either T-2 toxin (●) or trichodermin (○). Samples (50 µl) were removed, and radioactivity was determined as described in the Experimental section.

Fig. 4. Inhibition of polyphenylalanine synthesis in run-down reticulocyte lysates by trichodermin added at various times after commencing incorporations

Incorporation mixtures were prepared and programmed with poly(U) at a concentration of 200 $\mu\text{g}/\text{ml}$. Incubation was at 20°C and 50 μl samples were withdrawn as indicated in the Figure. Trichodermin, final concentration 50 $\mu\text{g}/\text{ml}$, was added to incorporation mixtures at times indicated in the Figure by vertical arrows. Experimental conditions were otherwise as described in the Experimental section. Control, $\bullet$; incorporation minus poly(U), ∇ ; addition of trichodermin at: 0 min, ∇ ; 5 min, Δ ; 8 min, $\blacksquare$; 12 min, $\triangle$; 18 min, $\square$; 25 min, $\circ$.

in vivo and *in vitro* in so far as it causes 'run off' of polyribosomes (Cundliffe *et al.*, 1974). Verrucaric acid is a potent inhibitor (more so even than trichodermin) of the reaction between puromycin and C-A-C-C-A-Leu-Ac as catalysed by yeast and human tonsil ribosomes (Carrasco *et al.*, 1973). Verrucaric acid can clearly gain efficient access to its ribosomal receptor site (presumably the peptidyl transferase centre) under the conditions used in the fragment reaction, where significantly the ribosomes carry no nascent polypeptide chains.

We have attempted to elucidate the situation with respect to T-2 toxin in the following way. Reticulocyte lysates were allowed to synthesize polyphenylalanine chains under the direction of poly(U), and at selected times trichodermin (final concentration 50 $\mu\text{g}/\text{ml}$) or T-2 toxin (final concentration 40 $\mu\text{g}/\text{ml}$) were added to the system and the kinetics of inhibition studied. The results are illustrated in Figs. 4 and 5. Trichodermin added at 5, 8, 12, 18 or 25 min after the start of the incubation caused inhibition of phenylalanine incorporation (Fig. 4). Kinetics of inhibition at all time-points were similar and a linear rate of inhibition (approx. 70%) was established shortly after drug addition. A similar procedure with T-2 toxin gave very different results (Fig. 5). The drug inhibited by the expected 50% (cf. Fig. 3) when added at early times after the start of the incubation. However, after a certain period (6–7 min incubation) the drug abruptly failed to inhibit phenylalanine incorporation, and incubation mixtures with and without T-2 toxin gave identical kinetics. This result therefore substantiates

Fig. 5. Inhibition of polyphenylalanine synthesis in run-down reticulocyte lysates by T-2 toxin added at various times after commencing incorporations

Incorporation mixtures were prepared as described under Fig. 4. T-2 toxin (40 $\mu\text{g}/\text{ml}$) was added at the times indicated in the Figure by the vertical arrows. Samples were removed and processed for radioactivity as described in the Experimental section. Control incorporation, $\blacksquare$; incorporation in the presence of dimethyl sulphoxide (final concentration 0.5%, v/v), $\bullet$; incorporation in the presence of T-2 toxin (40 $\mu\text{g}/\text{ml}$), $\circ$. The drug was dissolved in dimethyl sulphoxide, which was present in incorporation mixtures at a final concentration of 0.5% (v/v). (a)–(e) T-2 toxin added at 0, 4, 6, 8 and 10 min respectively, after commencing incubation. Control incubation contained equivalent amounts of dimethyl sulphoxide added at the same times as the drug.

the hypothesis that once ribosomes carry nascent polypeptide chains above a certain critical chain length T-2 toxin fails to inhibit the peptidyl transferase centre.

We are unable to state with any certainty what this chain length is. Polyphenylalanine is notoriously insoluble and methods for separating chains containing more than four phenylalanine residues pose considerable technical difficulty. We have, however, recovered ribosomes carrying ^{14}C -labelled nascent polyphenylalanine chains at the time (6–7 min) where T-2 toxin fails to inhibit poly(U)-directed incorporation and have subsequently cleaved off the peptides at high pH (Gilbert, 1963). Chromatographic analysis of such preparations (Bretthauer & Golichowski, 1967) has indicated that the average chain length of the polyphenylalanine is greater than four residues. In view of the short incubation time (6–7 min) and the low incubation temperature (20°C), we consider it unlikely that the polyphenylalanine chains would have a length greatly in excess of this number of residues.

Since our results suggest that T-2 toxin is excluded from functional interaction with its ribosomal receptor site by the presence of nascent polypeptide chains, the drug cannot be regarded as a specific inhibitor of a step unique to the initiation process itself. It would be better to consider T-2 toxin as an inhibitor of peptidyl transferase reactions in general with the important proviso that the conditions prevailing at the time of drug addition should allow T-2 toxin to gain access to this catalytic centre. The precise chemical reason(s) as to why trichodermin and T-2 toxin have different affinities for their ribosomal receptor site(s) under certain conditions remain open to speculation. However, our results in no way undermine the original suggestion by Cundliffe *et al.* (1974) that the nature of the substituent on C-15 may be of particular significance in determining the precise mode(s) of action of the 12,13-epoxytrichothecenes.

Thus, in trichodermin C-15 is part of an unsubstituted methyl group, whereas T-2 toxin has $-\text{CH}_2-\text{O}-\text{CO}-\text{CH}_3$ in this position on the molecule.

In conclusion we point out that the presence of nascent peptides bound to ribosomes through tRNA also apparently interferes with the functional binding of even trichodermin to its receptor site, although this interference is limited (Carter *et al.*, 1976). Thus, although the drug is an effective inhibitor of ribosomes carrying nascent polypeptide chains, it nevertheless finds easier access to its target site in the absence of such chains.

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References

- Bamburg, J. R. & Strong, F. M. (1971) *Microb. Toxins* **7**, 207–292
- Bretthauer, R. K. & Golichowski, A. M. (1967) *Biochim. Biophys. Acta* **155**, 549–557
- Carrasco, L., Barbacid, M. & Vazquez, D. (1973) *Biochim. Biophys. Acta* **312**, 368–376
- Carter, C. J., Cannon, M. & Smith, K. E. (1976) *Biochem. J.* **154**, 171–178
- Cundliffe, E., Cannon, M. & Davies, J. E. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 30–34
- Gilbert, W. (1963) *J. Mol. Biol.* **6**, 389–403
- Grollman, A. P. & Huang, M. T. (1970) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **29**, 537
- Schindler, D. (1974) *Nature (London)* **249**, 38–47
- Smith, K. E., Cannon, M. & Cundliffe, E. (1975) *FEBS Lett.* **50**, 8–12
- Ueno, Y. (1970) *Proc. U.S. Jpn. Conf. Toxic Micro-org.* **1st**, 76–79
- Wei, C. M., Hansen, B. S., Vaughan, M. H., Jr. & McLaughlin, C. S. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 713–717