The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis

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The effect of Shiga toxin, from *Shigella dysenteriae* 1, on the component reactions of peptide elongation were investigated. Enzymic binding of [³H]phenylalanine-tRNA to reticulocyte ribosomes was inhibited by 50% at 7 nM toxin. Elongation factor 1 (eEF-1)-dependent GTPase activity was also inhibited. Both reactions were not restored by addition of excess eEF-1 protein. In contrast, toxin concentrations of 200 nM were required to inhibit by 50% the elongation factor 2 (eEF-2)-dependent translocation of aminoacyl-tRNA on ribosomes. Addition of excess eEF-2 restored translocation activity. The eEF-2-dependent GTPase activity was unaffected at toxin concentrations below 100 nM, and Shiga-toxin concentrations of up to 1000 nM did not affect either GTP•eEF-2•ribosome complex-formation or peptidyltransferase activity. Thus Shiga toxin closely resembles alpha-sarcin in action, both being primary inhibitors of eEF-1-dependent reactions. In contrast, the 60 S ribosome inactivators ricin and phytolectin are primary inhibitors of eEF-2-dependent reactions of peptide elongation.

INTRODUCTION

Shiga toxin, the protein toxin of *Shigella dysenteriae* 1, has been implicated in diarrheal and dysentery disease and is one of the more potent bacterial toxins known [1,2]. Other *Shigella* species appear to produce this same toxin [3,4]. An immunologically similar toxin has been observed in various enteropathogenic strains of *Escherichia coli* [5], in several *Vibrio cholerae* and *Vibrio parahaemolyticus* strains [6], and in *E. coli* associated with haemolytic uraemic syndrome [7].

Shiga toxin has been purified from *Shigella dysenteriae* 1 by several groups [8–11]. Structural studies have shown the 68 kDa holotoxin to consist of a single 30 kDa protein and multiple 5 kDa subunit proteins [10,12]. The toxin inactivates cellular [13] and cell-free [10,11,13,14] protein synthesis by inhibition of peptide elongation [14]. Whereas the intact 58 kDa toxin is required for inhibition of protein synthesis in whole cells [10,13], the 30 kDa subunit alone is responsible for this activity in cell-free protein-synthesis systems [10,15]. It has also been established that Shiga toxin selectively inactivates 60 S ribosomal subunits [15].

The peptide-elongation cycle consists of three reactions: aminoacyl-tRNA binding, peptidyltransferase activity and translocation. Studies performed with unfractionated reticulocyte lysates have provided indirect evidence that Shiga-toxin inhibition of elongation occurs by inactivation of the aminoacyl-tRNA-binding step [16]. To define further the process by which Shiga toxin inhibits peptide elongation, we have investigated the direct effect of the toxin on eEF-1, eEF-2 and peptidyltransferase reactions in defined translation mixtures using purified eukaryotic factors and ribosomes. Portions of this work have been presented as preliminary communications [17–19].

EXPERIMENTAL

Activation of Shiga toxin

Shiga toxin was purified from *Shigella dysenteriae* 1 strain 3818-0 as described by Brown *et al.* [9]. It was activated, to increase potency *in vitro* by 70–100-fold [14], by adding 10 µg of tosylphenylalanylchloromethane-treated trypsin to a 1.0 ml solution containing 10 mM-Tris/HCl, pH 8.0, 100 mM-NaCl and Shiga toxin (0.42 mg/ml). The mixture was incubated at 37 °C for 60 min, at which time 0.02 µg of phenylmethanesulphonyl fluoride was added to inactivate the trypsin. Urea and dithiothreitol were added (final concns. 8 M and 10 mM respectively) and the mixture was incubated at 37 °C for 60 min. This sample was dialysed against 100 vol. of 18 MΩ-resistance water with three changes. Activated toxin was then freeze-dried and stored at –70 °C. Upon reconstitution in water, toxin prepared in this manner retained full activity as an inhibitor of cell-free protein synthesis.

Reticulocyte lysates and ribosomes

To obtain reticulocytes, New Zealand White rabbits (2–2.5 kg) were injected subcutaneously daily on days 1–4 with 0.25 ml of 2.5% phenylhydrazine (pH 7.0 in 0.14 M-NaCl)/kg body wt. On day 7 rabbits were heavily anaesthetized intramuscularly with 11 mg of ketamine hydrochloride and 2.2 mg of xylazine per kg body wt. Blood was then drained from the heart into freshly prepared ice-cold NKM solution (0.14 M-NaCl, 0.03 M-KCl, 0.002 M-MgCl₂) containing 200 units of heparin/ml. Reticulocytes as measured with Methylene Blue staining were found to represent ≥ 90% of the total cell population. This whole blood was filtered through cheesecloth, centrifuged at 1000 g for 10 min, and serum

was removed along with an upper 'buffy coat' layer of cells. Packed reticulocytes were gently resuspended in 20 vol. of NKM solution and centrifuged as above. This washing procedure was repeated a total of three times.

Lysate was prepared with the addition of 1 vol. of 18 M Ω -resistance water to packed cells, followed by gentle shaking (at 4 °C for 10 min), followed by centrifugation at 20000 *g* for 15 min. Portions of the resultant supernatant fluid were stored at -80 °C for up to 1 year without loss of activity. When first used, each batch of lysate was tested with various concentrations of haemin and magnesium acetate to determine those concentrations required for maximum protein-synthetic activity. Rates of protein synthesis in the lysate system were very close to that of whole reticulocytes.

Ribosomes were prepared from reticulocytes by washing with deoxycholate, and then with 0.5 M-KCl as described previously [20], and are referred to as 'DOC-KCl-washed ribosomes'. Ribosomal subunits were obtained by dissociation of polysomes in the presence of 0.5 M-KCl and 1 mM-puromycin [21,22]. Subunits were separated in 35 ml 10-30 % (w/v) sucrose [in TKMD 40/500/5/2 (40 mM-Tris/HCl, pH 7.4, 500 mM-KCl, 5 mM-MgCl₂, 2 mM-dithiothreitol)] gradients by centrifugation in a SW27 rotor (Beckman) at 13500 rev./min for 16 h at 4 °C and precipitated by addition of MgCl₂ to 10 mM and 0.7 vol. of pre-cooled (-20 °C) 95% (v/v) ethanol. Precipitated ribosomes were collected by centrifugation at 6000 *g* for 20 min at 4 °C, resuspended in TKMD 20/70/3/1 (20 mM-Tris/HCl, pH 7.4, 70 mM-KCl, 3 mM-MgCl₂, 1 mM-dithiothreitol) and stored at -70 °C.

Purification of eEF-1 and eEF-2 from rabbit reticulocytes

eEF-1 and eEF-2 proteins were isolated from the 100000 *g* supernatant of reticulocyte lysate as described previously [20,23]. The purification procedure included sequential steps of (NH₄)₂SO₄ fractionation, gel filtration, DEAE-cellulose, hydroxyapatite and CM-cellulose chromatography. In some cases, purified elongation factors from wheat germ supplied by Dr. J. Ravel (University of Texas at Austin) were utilized and found to be comparable in activity with the reticulocyte proteins.

[³H]Phenylalanine-tRNA synthesis

Aminoacylation of yeast tRNA with [³H]phenylalanine was carried out in a 4.0 ml reaction mixture containing 100 mM-Tris/HCl, pH 7.4, 10 mM-magnesium acetate, 10 mM-dithiothreitol, 2 mM-ATP, 2.6 mg of phosphocreatine, 0.1 mg of creatine kinase, 200 μ g of reticulocyte ribosomal 0.5 M-KCl-wash protein, 5 mg of tRNA and 100 μ g of [³H]phenylalanine (2000 μ Ci/ μ mol). The reaction mixture was incubated at 37 °C for 40 min and then monitored for cold 10%-trichloroacetic acid-insoluble radioactivity precipitated on to glass-fibre filters (GF/C, Whatman). [³H]Phenylalanine-tRNA was extracted by addition of 2.0 M-potassium acetate, pH 5.0, to a final concentration of 0.1 M and 1 vol. of phenol saturated with 10 mM-potassium acetate, pH 5.0. After agitation for 15 min at 4 °C and centrifugation at 5000 *g* for 20 min at 4 °C, the aqueous layer was removed and [³H]phenylalanine-tRNA precipitated by addition of 2.5 vol. of 95% ethanol at -20 °C for 2 h. The [³H]phenyl-

alanine-tRNA pellet was washed with 95% ethanol and then with diethyl ether to remove residual phenol.

[³H]Phenylalanine-tRNA binding to ribosomes

The 100 μ l reaction mixture contained, in order of addition: 25 mM-Tris/HCl, pH 7.4, 62 mM-KCl, 5 mM-magnesium acetate, 2.4 mM-dithiothreitol, 40 μ M-GTP, 20 μ g of poly(U), activated Shiga toxin in water added to final concentrations indicated in the text, 1.0 *A*₂₆₀ unit (21 pmol) of DOC-KCl-washed ribosomes, 1.0 μ g of eEF-1 protein and 5-10 pmol of [³H]phenylalanine-tRNA (2000 μ Ci/ μ mol). The reaction mixture was incubated at 37 °C for 5 min. Then 6 ml of ice-cold solution A (50 mM-Tris/HCl, pH 7.4, 50 mM-KCl, 8 mM-MgCl₂) was added to stop the reaction. The contents were applied to a nitrocellulose filter (BA85; 0.45 μ m pore size; Schleicher and Schuell), and the filters were washed with 3 $\times$ 15 ml of solution A, placed in a scintillation vial with 5 ml of aqueous scintillation cocktail, shaken for 60 min at 4 °C and counted for radioactivity.

Assay of eEF-1 and eEF-2 GTPase

The eEF-1 GTPase reaction [16] mixture was performed in a total volume of 150 μ l containing, in order of addition: 25 mM-Tris/HCl, pH 7.4, 100 mM-KCl, 5 mM-magnesium acetate, 2.5 mM-dithiothreitol, 5 μ g of poly(U), Shiga toxin as indicated, 1.0 *A*₂₆₀ unit of DOC-KCl-washed ribosomes, 1.0 μ g of eEF-1, 15 μ g of unlabelled phenylalanine-tRNA (20 pmol) and 5 μ M- $[\gamma$ -³²P]GTP (1000 μ Ci/ μ mol). The reaction mixture was incubated at 37 °C for 10 min, stopped by addition of 0.25 ml of 0.02 M-silicotungstic acid in 0.01 M-H₂SO₄, 0.5 ml of 1 mM-potassium phosphate, pH 6.8, and 0.25 ml of 5% (w/v) ammonium molybdate in 2 M-H₂SO₄. The phosphomolybdate was extracted into 1.0 ml of 2-methylpropan-1-ol/benzene (1:1, v/v), centrifuged at 500 *g* for 5 min, and radioactivity of 0.5 ml of the aqueous phase was monitored in a scintillation counter.

eEF-2 GTPase was measured in a 50 μ l reaction mixture. The order of addition of components was identical with that for eEF-1 GTPase described above, except that 0.2 μ g of eEF-2 protein was added as indicated in place of eEF-1 protein; phenylalanine-tRNA and poly(U) were omitted, and the reaction was incubated at 37 °C for 20 min.

eEF-2 translocation assay

[³H]Phenylalanine-tRNA was non-enzymically bound to DOC-KCl-washed ribosomes in a batch reaction containing the following, in a final total volume of 52 ml: 50 mM-Tris/HCl, pH 7.4, 120 mM-KCl, 16 mM-MgCl₂, 5 mM-dithiothreitol, 10 mg of poly(U), 620 *A*₂₆₀ units of DOC-KCl-washed ribosomes, and 3 nmol of [³H]phenylalanine-tRNA (1500 μ Ci/ μ mol). The reaction mixture was incubated at 37 °C for 20 min and chilled on ice for 10 min. Approx. 26 ml of the reaction mixture was layered over 7 ml of 15% sucrose solution containing 50 mM-Tris/HCl, pH 7.4, 120 mM-KCl, 8 mM-MgCl₂ and 5 mM-2-mercaptoethanol in a 35 ml tube. The contents were centrifuged in a SW-27 rotor (Beckman) at 24000 rev./min for 16 h at 4 °C. Supernatants were decanted and the pellets resuspended in 20 mM-Tris/HCl

Fig. 1. Effect of Shiga toxin on total protein synthesis, eEF-1-dependent [^3H]phenylalanyl-tRNA binding and eEF-1 GTPase activity

Total protein synthesis (●) was measured by monitoring [^3H]leucine incorporation into trichloroacetic acid-insoluble reticulocyte lysate protein. Codon-directed binding of [^3H]phenylalanyl-tRNA to 80 S ribosomes (■) was carried out in the presence of eEF-1 protein as described in the Experimental section. eEF-1 GTPase activity (○) with 0.5 M-KCl-washed reticulocyte ribosomes was monitored in the presence of [$\gamma\text{-}^{32}\text{P}$]GTP (see the Experimental section). Control (100%) values for the reactions were 13400, 3587 and 750 c.p.m. respectively.

(pH 7.4)/100 mM-KCl/5 mM-MgCl₂/1 mM-2-mercaptoethanol/10% (v/v) glycerol at 125 A_{260} units/ml.

The translocation assay contained the following, listed in order of addition, in a final volume of 0.5 ml: 50 mM-Tris/HCl, pH 7.4, 70 mM-KCl, 5 mM-MgCl₂, 5 mM-dithiothreitol, 3.0 A_{260} units of DOC-KCl-washed ribosomes with [^3H]phenylalanine-tRNA (5000 c.p.m.) non-enzymically bound as described above, activated Shiga toxin as indicated, 0.5 μg of eEF-2 protein and 0.2 mM-GTP. The reaction mixture was incubated at 37 °C for 6 min and cooled at 4 °C. Puromycin hydrochloride was added to 1 mM and incubated at 4 °C for 20 min. [^3H]Phenylalanine-puromycin was extracted from the reaction mixture by addition of 0.5 ml of 2 M-NH₄HCO₃, pH 9.0, and 1.0 ml of ethyl acetate. A portion of the organic phase was monitored for radioactivity in 10 ml of scintillation fluid.

[eEF-2·GTP·80 S ribosome] complex-formation

The 100 μl reaction mixture contained the following components, listed in order of addition: 25 mM-Tris/HCl, pH 7.4, 110 mM-potassium acetate, 5 mM-magnesium acetate, 2.5 mM-dithiothreitol, 1.0 A_{260} unit of DOC-KCl-washed ribosomes (21 pmol), 0.4 μg

of eEF-2 protein, Shiga toxin as indicated, and 1 μM -[^3H]GTP (5000 $\mu\text{Ci}/\mu\text{mol}$). The reaction was incubated at 37 °C for 10 min, terminated by addition of 6 ml of ice-cold Solution A, and collected on a BA85 0.45 μm -pore-size nitrocellulose filter. Filters were washed with 3 $\times$ 15 ml of ice-cold Solution A, placed in vials containing 5 ml of aqueous scintillant, shaken for 60 min at 4 °C and counted for radioactivity.

RESULTS

Effect of Shiga toxin on eEF-1-dependent reactions

Enzymic binding of [^3H]phenylalanine-tRNA to DOC-KCl-washed ribosomes was inhibited by Shiga toxin in a concentration-dependent fashion (Fig. 1); 50% inhibition of eEF-1-dependent binding was observed at 7 nM toxin. Enzymic phenylalanine-tRNA binding remained maximally inhibited at 40% of control values (Fig. 1). The toxin, activated by trypsin, urea and dithiothreitol treatment, inhibited reticulocyte-lysate endogenous pro-

Fig. 2. Effect of eEF-1 protein concentration on Shiga-toxin inhibition of enzymic [^3H]phenylalanyl-tRNA binding to ribosomes

Reaction conditions were the same as those described in Fig. 1. (a) Incubations carried out in the absence (○) or presence (●) of 1 μM -Shiga toxin and (b) data presented as percentage of uninhibited control values at each EF-1 concentration (■).

Table 1. Effect of Shiga toxin on eEF-1-dependent phenylalanyl-tRNA binding and GTPase activities

Binding of [³H]phenylalanyl-tRNA to 0.5 M-KCl-washed reticulocyte ribosomes and eEF-1 GTPase activities were monitored as described in Fig. 1 and the Experimental section. Toxins were added to reaction mixtures to yield the final concentrations indicated. Data are presented as c.p.m. and as percentage incorporation compared with complete reactions performed in the absence of inhibitors: N.D., not determined.

Toxin	Inhibitor	Phenylalanyl-tRNA binding		GTPase activity	
	Concn. (μ M)	(c.p.m.)	(% of control)	(c.p.m.)	(% of control)
None	—	3477	100	810	100
Shiga toxin	0.6	1022	29	N.D.	N.D.
	1.3	N.D.	N.D.	400	49
Alpha-sarcin	0.25	1580	45	N.D.	N.D.
	1.25	N.D.	N.D.	0	0
Phytolaccin	0.75	N.D.	N.D.	542	67
	1.1	1390	40	N.D.	N.D.
Diphtheria toxin	0.3	3714	107	795	98

tein synthesis by 50% at 6.4 nM concentration (Fig. 1), in close correlation with the effect on enzymic binding of [³H]phenylalanine-tRNA.

Excess eEF-1 has been shown to reverse partially the inhibitory effect of ricin and phytolaccin ('PAP', *Phytolacca* antiviral peptide) on peptide elongation [24–26]. Thus we tested for a possible effect of excess eEF-1 on Shiga-toxin inhibition of phenylalanine-tRNA binding to ribosomes. In control reactions, maximum enzymic binding of [³H]phenylalanine-tRNA was obtained at 60 μ g of eEF-1 protein/ml (Fig. 2a). In Shiga-toxin-treated preparations, increasing eEF-1 concentrations up to 90 μ g/ml did not overcome the inhibition of binding. In fact, in the presence of Shiga toxin phenylalanine-tRNA binding was decreased further, from 50% to 30% of control values, as eEF-1 protein was increased (Fig. 2b). This may be due to the increased efficiency of Shiga toxin at higher rates of the reaction.

Phenylalanyl-tRNA binding to DOC-KCl-washed ribosomes was measured at ≥ 10 mM-Mg²⁺ to test the effect of Shiga toxin on non-enzymic binding. Whereas eEF-1-dependent phenylalanyl-tRNA binding carried out in 6 mM-Mg²⁺ is sensitive to Shiga toxin (see above), non-enzymic binding performed at ≥ 10 mM-Mg²⁺ was refractory to toxin action (results not shown). These data suggest that Shiga toxin may not be able to interact with ribosomes at higher Mg²⁺ concentrations. Similar results have been obtained with phytolaccin [27] and ricin A [28].

To define further the inhibition of eEF-1-associated reactions, the effect of Shiga toxin on eEF-1-dependent GTPase activity was examined. GTPase activity was measured during incubation of DOC-KCl-washed ribosomes, phenylalanyl-tRNA and eEF-1 protein with different concentrations of activated toxin (Fig. 1). Shiga toxin exhibited a marked inhibitory effect on eEF-1 GTPase activity in a concentration-dependent fashion within the 2–130 nM toxin range. GTPase activity was decreased to 50% of control values by 130 nM Shiga toxin. No further increase in inhibition was observed at a 10-fold higher toxin concentration. Data presented in Fig. 1 indicate that eEF-1 GTPase and enzymic phenylalanyl-tRNA binding both become inhibited at

Shiga-toxin concentrations which inhibit reticulocyte total protein synthesis.

The effect of Shiga toxin on enzymic [³H]phenylalanyl-tRNA binding to ribosomes was compared with that of other toxins which also inhibit this reaction. Alpha-sarcin and phytolaccin (previously referred to as 'PAP'; [27]) inhibited [³H]phenylalanyl-tRNA binding to ribosomes as did Shiga toxin; diphtheria toxin was without effect (Table 1). Thus Shiga toxin, alpha-sarcin and phytolaccin specifically inactivate 60 S ribosomes and are also inhibitors of the eEF-1-dependent aminoacyl-tRNA-binding reaction. In contrast, diphtheria toxin, which inactivates eEF-2 protein by ADP-ribosylation, had no effect on eEF-1-dependent [³H]phenylalanyl-tRNA binding to ribosomes.

We also compared the inhibition of eEF-1 GTPase activity by Shiga toxin with the effect of alpha-sarcin, phytolaccin and diphtheria toxin on this reaction (Table 1). At saturating concentrations, Shiga toxin decreased GTPase activity to 49% of the control value. In contrast, diphtheria toxin had no effect on this eEF-1-dependent reaction, whereas alpha-sarcin completely inhibited, and phytolaccin decreased, the activity to 67% of control. Thus Shiga toxin has in common with alpha-sarcin and phytolaccin the ability to inhibit eEF-1-dependent GTPase activity [24,27].

Effect of Shiga toxin on peptidyltransferase

To test whether Shiga toxin inhibited peptidyltransferase, we employed an assay in which the eEF-2-dependent translocation and peptidyltransferase reactions proceed in two separate but sequential steps. [³H]-phenylalanyl-tRNA is bound non-enzymically into the ribosomal A-site. The first step involves the eEF-2-dependent movement of [³H]phenylalanine-tRNA on ribosomes from the A- to the P-site. The second incubation involves reaction by peptidyltransferase of the [³H]-phenylalanyl-tRNA located in the P-site with puromycin to yield [³H]phenylalanyl-puromycin. When 1 μ M Shiga toxin was present during the peptidyltransferase step (i.e. the second incubation), no effect on that activity was detected (Table 2). In contrast, 1 μ M Shiga toxin added during the translocation step (i.e. the first incubation)

Table 2. Effect of Shiga toxin on eEF-2-dependent translocation and peptidyltransferase reactions

Translocation of non-enzymically bound [^3H]phenylalanyl-tRNA on reticulocyte ribosomes was performed as described in the Experimental section. Translocation of [^3H]phenylalanyl-tRNA from the A- to the P-site on ribosomes was carried out during the first incubation in the presence of eEF-2 protein and GTP. The reaction mixture was then cooled at 4 °C, puromycin was added and [^3H]phenylalanyl-puromycin formation allowed to proceed in the second incubation. Inhibitors were added in either the first or the second incubation as indicated below. Control (100%) formation of [^3H]phenylalanyl-puromycin was 2236 c.p.m.

First incubation	Second incubation	[^3H]Phenylalanyl-puromycin formed (% of control)
—	—	10
Shiga toxin (1 μM)	—	45
—	Shiga toxin (1 μM)	100
Alpha-sarcin (1 μM)	—	65
—	Alpha-sarcin (1 μM)	87
Phytolaccin (1 μM)	—	23
—	Phytolaccin (1 μM)	92
Cycloheximide (1 mM)	—	56
—	Cycloheximide (1 mM)	98

decreased [^3H]phenylalanyl-puromycin formation to 45% of control values (see below). Other inhibitors of the translocation reaction, including alpha-sarcin, phytolaccin and cycloheximide, also inhibited translocation, but exhibited only a marginal direct effect on peptidyltransferase activity (Table 2).

Effect of Shiga toxin on eEF-2-dependent reactions

The effect of Shiga toxin on eEF-2-dependent reactions was assessed by examination, in defined reaction mixtures, of (1) aminoacyl-tRNA translocation, (2) eEF-2-dependent GTPase activity and (3) GTP-eEF-2-ribosome complex-formation. eEF-2-dependent aminoacyl-tRNA translocation was performed by using the assay described above, except that both eEF-2 and puromycin were present together throughout. Ribosomes were prepared with [^3H]phenylalanine-tRNA non-enzymically bound into the ribosomal A-site. These ribosomes were incubated with toxin, eEF-2 protein, GTP and puromycin. Translocation activity was monitored by formation of [^3H]phenylalanyl-puromycin. As shown in Fig 3, Shiga toxin inhibited the translocation reaction only when present at concentrations above 10 nM. At 640 nM, Shiga toxin decreased translocation activity to 20% of the control value. It should be noted that the concentration of Shiga toxin required to inhibit translocation by 50% was > 50-fold higher than that needed to inhibit enzymic binding of [^3H]phenylalanyl-tRNA and total protein synthesis by 50% (Figs. 1 and 3).

Excess eEF-2 protein completely reversed Shiga-toxin inhibition of the translocation reaction (Fig. 4). Translocation reactions were saturated by 0.6 μg of eEF-2 protein/ml in the absence of toxin, but in the presence of Shiga toxin required 6.0 μg of eEF-2 protein/ml to reach maximum translocation (Fig. 4). These data suggest that Shiga toxin may alter the affinity of ribosomes for eEF-2 protein, a phenomenon which is completely overcome by excess eEF-2 protein. In this translocation reaction, it was also determined that varying the KCl concentration over the range 10–130 mM had only a slight effect on Shiga toxin's ability to inhibit [^3H]phenylalanyl-puromycin formation (results not shown). Thus, in the presence of limiting eEF-2 protein, increasing the rate of translocation by increasing the KCl concentration had

Fig. 3. Effect of Shiga-toxin concentration on eEF-2-dependent translocation of aminoacyl-tRNA and eEF-2 GTPase activity

Enzymic translocation of non-enzymically bound [^3H]phenylalanyl-tRNA on ribosomes (■) was carried out in the presence of eEF-2 protein and monitored as [^3H]phenylalanyl-puromycin formation (see the Experimental section). eEF-2-dependent GTPase activity (○) was measured in reactions containing ribosomes and [γ - ^{32}P]GTP as described in the Experimental section. Total protein synthesis (●) was carried out in reticulocyte lysates as described in Fig. 1. Control 100% values for the three reactions were 2340, 6700 and 13400 c.p.m. respectively.

little effect on the ability of Shiga toxin to prevent eEF-2 interaction with ribosomes.

The effect of Shiga toxin on eEF-2-dependent GTPase activity was examined over a concentration range of

Fig. 4. Effect of eEF-2 protein concentration on Shiga-toxin inhibition of aminoacyl-tRNA translocation on ribosomes

Reactions were performed in the absence (○) or presence (●) of 1 μ M-Shiga toxin as described in Fig. 3.

0.1 nM to 1 μ M toxin (Fig. 3). This GTPase activity is considered to be 'uncoupled', as it was carried out in the presence of ribosomes, GTP and eEF-2 protein, but in the absence of aminoacyl-tRNA and mRNA. Although 50% inhibition of lysate protein synthesis occurs at 6.4 nM Shiga toxin, only slight inhibition of GTPase activity was observed at concentrations of up to 1 μ M toxin. As observed with inhibition of the translocation reaction, excess eEF-2 protein also reversed the minimal effect of the toxin on eEF-2 GTPase activity (results not shown).

Another means of measuring eEF-2 interaction with ribosomes is to monitor formation of the ternary ribosomal complex comprising GTP, eEF-2 protein and ribosomes. Both aminoacyl-tRNA and mRNA are omitted from this assay. Shiga toxin at concentrations up to 1 μ M did not affect complex-formation (Table 3). Thus Shiga toxin appears to differ from other ribosome-inactivating toxins such as phytolaccin and abrin, which are strong inhibitors of ternary-complex formation (Table 3; references [24] and [27]).

DISCUSSION

In this investigation, we examined the effect of Shiga toxin on defined peptide-elongation reactions of eukaryotic protein synthesis. Our results demonstrate that the primary functional lesion induced by Shiga toxin is a direct inhibition of eEF-1-dependent aminoacyl-tRNA binding to ribosomes. The present results show that Shiga toxin inhibits both enzymic [3 H]phenylalanyl-tRNA binding to ribosomes and eEF-1-dependent GTPase activity at toxin concentrations similar to those required for inhibition of overall protein synthesis in reticulocyte lysates and that the inhibition of [3 H]phenylalanyl-tRNA binding was not overcome by increased concentrations of eEF-1 protein. Peptidyltransferase activity was not affected. In contrast with eEF-1-dependent aminoacyl-tRNA binding, a more than 20-fold higher toxin concentration was required to inhibit the translocation process by 50%. Increased concentrations of eEF-2 restored translocation activity in full. Moreover, effects of Shiga toxin on both eEF-2-dependent GTPase and [3 H]GTP·eEF-2·ribosome complex-formation are negligible.

Table 3. Effect of Shiga toxin on formation of [3 H]GTP·eEF-2·ribosome complex

Ternary-complex formation, conducted in the presence of purified eEF-2 protein and 0.5 M-KCl-washed ribosomes, was monitored by collection on nitrocellulose filters as described in the Experimental section. Toxins were added to reactions to yield the final concentrations indicated. Data are presented as percentage incorporation compared with complete reaction mixtures performed in the absence of inhibitors

Addition	[3 H]GTP·eEF-2·ribosome complex-formation	
	(c.p.m.)	(% of control)
Control	5312	100
Shiga toxin (0.1 nM)	5283	99
Shiga toxin (10 nM)	5205	98
Shiga toxin (1 μ M)	5011	94
Phytolaccin (0.1 μ M)	720	13

These data, combined with previous findings, begin to describe a detailed picture of the effect of Shiga toxin on ribosome function. We have previously demonstrated that the peptide-initiation process is unaffected by Shiga toxin [16]. Similarly, Shiga toxin does not inhibit aminoacylation of tRNA [29] or peptidyltransferase reactions on eukaryotic ribosomes [15]. Studies conducted with crude reticulocyte lysate have provided indirect evidence that Shiga toxin exhibits a primary effect on aminoacyl-tRNA binding to ribosomes [16].

Shiga toxin is the first bacterial-derived toxin to be described which resembles other ribosome-inactivating catalytic protein toxins, such as ricin, abrin, phytolaccin and alpha-sarcin. All of these toxins inhibit protein synthesis as a result of direct action on the 60 S ribosomal subunit [28]. However, our results also reveal a major difference between Shiga toxin and the other toxins. eEF-1-dependent binding of aminoacyl-tRNA to ribosomes appears to be the primary reaction inhibited by Shiga toxin. Although the other toxins may inhibit this step, they appear to affect preferentially eEF-2-dependent translocation reactions [30–32]. These findings suggest that ribosomes inactivated by Shiga toxin have a much decreased affinity for eEF-1 protein, whereas ribosomes inactivated by ricin or phytolaccin exhibit a much decreased affinity for eEF-2 protein. This would help to explain why these specifically inhibited reactions are not easily reversed by addition of excess of the corresponding elongation factor.

It is still possible that a large excess of eEF-1 protein in whole cells could prevent Shiga-toxin inhibition of aminoacyl-tRNA binding to ribosomes. However, our calculations indicate that conditions in the [3 H]phenylalanyl-tRNA-binding assay of the present study closely match the relative amounts of eEF-1 protein and ribosomes found in crude reticulocyte lysate [33]. In both cases, eEF-1 protein is present in a 25-fold molar excess compared with ribosomes. Therefore we must conclude that Shiga toxin would indeed be a potent inhibitor of [3 H]phenylalanyl-tRNA binding to ribosomes in whole cells.

Shiga toxin is a less potent inhibitor of the

eEF-2-dependent translocation reaction. Conditions for this defined reaction in the present study actually favoured toxin inhibition of translocation, i.e. under conditions where eEF-2 protein was limiting. The molar ratio of eEF-2 to ribosomes was 1:3 in the defined reaction as compared with a 1:1 ratio normally present in reticulocyte lysates [33]. Thus our data suggest that Shiga toxin would have a very limited inhibitory effect on eEF-2-dependent reactions in reticulocyte lysates and whole cells. Indeed, indirect measurements of eEF-2 reactions in such lysates have been carried out and confirm this concept [16].

Shiga toxin also differs from the other ribosome-inactivating toxins at the level of peptide initiation. Ribosomes inactivated by Shiga toxin remain capable of completing initiation complexes [16]. In contrast, abrin was shown to be a moderate inhibitor of the final step of initiation, i.e. binding of the 60 S ribosomal subunit to the growing initiation complex [34], and ricin altered whole-cell polysome profiles in a manner indicative of a preferential inhibition of peptide initiation [35]. Only phytolaccin appears to resemble Shiga toxin more closely in this regard, as both toxins inhibit elongation without affecting initiation [27].

Having established directly that Shiga toxin causes a specific functional lesion in peptide elongation, we also need to answer the question of how Shiga toxin inactivates 60 S ribosomes. It is unlikely that Shiga or other similar toxins must bind firmly and stoichiometrically to ribosomes for continued inactivation. To the contrary, all data available indicate that these toxins inactivate 60 S ribosomes catalytically. To date, we and others have failed to detect a change in any of the 47 ribosomal proteins or three rRNA species after inactivation of 60 S ribosomes by ricin, phytolaccin or Shiga toxin. The two exceptions are alpha-sarcin and colicin E3, which were shown to be RNAases specific for 28 S and 16 S rRNAs of intact 60 S and 30 S ribosomes, respectively [36–38]. More recently we have observed that Shiga toxin as well as ricin and phytolaccin preparations possess a RNAase activity using free 5.8 S rRNA as a substrate [39]. Shiga toxin appears to prefer hydrolysis of single-stranded regions located in hairpin loops of 5.8 S rRNA [39]. Other data indicate that, at the temperature at which Shiga toxin is heat-denatured, there is a concomitant loss of both protein-synthesis-inhibitory and RNAase activities [39]. Further characterization of this RNAase activity may help to reveal the specific structural lesion associated with Shiga-toxin inactivation of the 60 S subunit.

It would be helpful to know how eEF-1 and eEF-2 interact with 60 S ribosomes during peptide elongation, as it is our working hypothesis that Shiga toxin causes a structural change in the proximity of the ribosomal binding site for eEF proteins. Evidence has been presented that acidic ribosomal proteins related to *E. coli* L7/L12 ribosomal proteins are required for eEF-1 and eEF-2-dependent reactions on 60 S ribosomes [40,41]. More recently, others have shown that reticulocyte eEF-Tu, a 53000 Da basic protein, contains a site which binds G-rich ribonucleotides such as 28 S rRNA, resulting in an enhanced GTPase activity of the eEF-Tu protein [33,42,43]. Therefore it would seem appropriate that efforts be directed towards a further elucidation of Shiga-toxin interaction with rRNA species of 60 S ribosomes.

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REFERENCES

1. van Heyningen, W. E. (1971) in *Microbial Toxins*, vol. 2 (Kadis, S., Montie, T. C. & Ajl, S. J., eds.), pp. 255–269, Academic Press, New York
2. Cantey, J. R. (1985) *J. Infect. Dis.* **151**, 766–771
3. O'Brien, A. D., Thompson, M. R., Gemski, P., Doctor, B. P. & Formal, S. B. (1977) *Infect. Immun.* **15**, 796–798
4. Keusch, G. T. & Jacewicz, M. (1977) *J. Infect. Dis.* **135**, 552–556
5. O'Brien, A. D., LaVeck, G. D., Thompson, M. R. & Formal, S. B. (1982) *J. Infect. Dis.* **146**, 763–769
6. O'Brien, A. D., Chen, M. E., Holmes, R. K., Kaper, J. & Levine, M. M. (1984) *Lancet* **i**, 77–78
7. Karmali, M. A., Petric, M., Lim, C., Fleming, P. C., Arbus, G. S. & Lior, H. (1985) *J. Infect. Dis.* **5**, 775–782
8. O'Brien, A. D., LaVeck, G. D., Griffin, D. E. & Thompson, M. R. (1980) *Infect. Immun.* **30**, 170–179
9. Brown, J. E., Griffin, D. E., Rothman, S. W. & Doctor, B. P. (1982) *Infect. Immun.* **36**, 996–1005
10. Olsnes, S., Reisbig, R. & Eiklid, K. (1981) *J. Biol. Chem.* **256**, 8732–8738
11. Donohue-Rolfe, A., Keusch, G. T., Thorley-Lawson, D. & Jacewicz, M. (1984) *J. Exp. Med.* **160**, 1767–1781
12. O'Brien, A. D. & LaVeck, G. D. (1983) *Infect. Immun.* **40**, 675–683
13. Brown, J. E., Rothman, S. W. & Doctor, B. P. (1980) *Infect. Immun.* **29**, 98–107
14. Brown, J. E., Ussery, M. A., Leppla, S. A. & Rothman, S. W. (1981) *FEBS Lett.* **117**, 84–88
15. Reisbig, R., Olsnes, S. & Eiklid, K. (1981) *J. Biol. Chem.* **256**, 8739–8744
16. Brown, J. E., Oberg, T. G., Ussery, M. A. & Moran, T. P. (1986) *Microb. Pathog.* **1**, 325–334
17. Oberg, T. G., Moran, T. P. & Brown, J. E. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 1808
18. Oberg, T. G., Brown, J. E. & Moran, T. P. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 1953
19. Oberg, T. G., Brown, J. E. & Moran, T. P. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 674
20. Hardesty, B., McKeehan, W. & Culp, W. (1971) *Methods Enzymol.* **20**, 316–328
21. Blobel, G. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 924–928
22. Oberg, T. G., Culp, W. J., McKeehan, W. L. & Hardesty, B. (1971) *J. Biol. Chem.* **246**, 174–181
23. Hardesty, B. & McKeehan, W. (1971) *Methods Enzymol.* **20**, 315–330
24. Fernandez-Puentes, C. & Vazquez, D. (1977) *FEBS Lett.* **78**, 143–146
25. Irvin, J. D., Kelly, T. & Robertus, J. D. (1980) *Arch. Biochem. Biophys.* **200**, 418–425
26. Fernandez-Puentes, C., Carrasco, L. & Vazquez, D. (1976) *Biochemistry* **15**, 4363–4369
27. Oberg, T. G., Irvin, J. D. & Hardesty, B. (1973) *Arch. Biochem. Biophys.* **155**, 278–289
28. Olsnes, S. & Pihl, A. (1976) in *Receptors and Recognition, Series B: The Specificity and Action of Animal, Bacterial and Plant Toxins* (Cuatrecasas, P., ed.), pp. 130–173, Chapman and Hall, London
29. Thompson, M. R., Steinberg, M. S., Gemski, P., Formal, S. & Doctor, B. P. (1976) *Biochem. Biophys. Res Commun.* **71**, 783–788
30. Gessner, S. L. & Irvin, J. D. (1980) *J. Biol. Chem.* **255**, 3251–3253

31. Sperti, S., Montanaro, L., Mattioli, A., Testoni, G. & Stirpe, F. (1976) *Biochem. J.* **156**, 7–13
32. Grasmuk, H., Nolan, R. D. & Drews, J. (1977) *Eur. J. Biochem.* **79**, 93–102
33. Slobin, L. I. (1980) *Eur. J. Biochem.* **110**, 555–563
34. Skorve, J., Abraham, A. K., Olsnes, S. & Pihl, A. (1977) *Eur. J. Biochem.* **79**, 559–564
35. Grollman, A. P., Grunfeld, C., Brewer, C. F. & Marcus, D. M. (1974) *Cancer Chemother. Rep.* **58**, 491–501
36. Jakes, K. S. (1982) in *Molecular Action of Toxins and Viruses* (Cohen, P. & van Heyningen, S., eds.), pp. 131–168, Elsevier Biomedical, Amsterdam
37. Schindler, D. G. & Davies, J. E. (1977) *Nucleic Acids Res.* **4**, 1097–1110
38. Endo, Y. & Wool, I. G. (1982) *J. Biol. Chem.* **257**, 9054–9060
39. Obrig, T. G., Moran, T. P. & Colinas, R. J. (1985) *Biochem. Biophys. Res. Commun.* **130**, 879–884
40. Moller, W., Slobin, L. I., Amons, R. & Richter, D. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4744–4748
41. van Agthoven, A. J., Maassen, J. A. & Moller, W. (1977) *Biochem. Biophys. Res. Commun.* **77**, 989–997
42. Slobin, L. I., Clark, R. V. & Olson, M. O. J. (1983) *Biochemistry* **22**, 1911–1917
43. Slobin, L. I. (1983) *J. Biol. Chem.* **258**, 4895–4900

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