INHIBITION OF PROTEIN SYNTHESIS BY SHIGA TOXIN

Activation of the toxin and inhibition of peptide elongation

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1. Introduction

Shigella dysenteriae 1 produces a toxin (Shiga toxin) which is lethal to mice and rabbits [1], enterotoxic in the rabbit ileal loop model [2] and cytotoxic to various cell lines [3–6]. Inhibition of protein synthesis and DNA synthesis by Shiga toxin occurs in intact cells prior to any effects on RNA synthesis or amino acid uptake and before perturbation of intracellular potassium concentration [7]. Studies with partially purified toxin have indicated that it can inhibit cell-free protein synthesis in both mammalian [8] and poly(U)-directed bacterial [9] extracts at relatively high toxin concentrations (μg/ml). In the bacterial system inhibition results from failure of polyuridylic acid to attach to ribosomes. Biosynthesis in the form of an inactive proenzyme is characteristic of several toxin proteins which inhibit protein synthesis, i.e., diphtheria toxin [10], *Pseudomonas aeruginosa* exotoxin A [11,12], abrin and ricin [13] and colicin E3 [14]. In view of the much smaller (pg) amounts of purified Shigella toxin needed to demonstrate cytotoxicity [5,7], we decided to investigate the possibility that it may exist as an inactive proenzyme. In this report we demonstrate that inhibition of cell-free protein synthesis by highly purified Shiga toxin is enhanced by prior activation of the protein. Inhibition of peptide elongation appears to be the primary mechanism by which activated toxin affects protein synthesis.

Abbreviations: poly(U), polyuridylic acid; DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; EF-2, polypeptide elongation factor 2; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

2. Materials and methods

Shiga toxin was purified from cell lysates of *Shigella dysenteriae* 1 strain 3818]0 [15]. Purification involved overnight high-speed centrifugation, ammonium sulfate fractionation, DEAE-cellulose and CM-cellulose chromatography, ammonium sulfate concentration, Sephacryl S-200 gel filtration, preparative isoelectric focusing and Sephadex G-25 chromatography. HeLa cell cytotoxicity was enriched ~8000-fold compared to the cell lysate. The purified toxin is lethal to mice and produces fluid secretion in rabbit ileal loops. By polyacrylamide gel electrophoresis, this preparation shows two closely migrating bands, each possessing cytotoxicity proportional to its staining intensity.

Cell-free protein synthesis was performed using either rabbit reticulocyte lysate or wheat germ extract and globin mRNA from Bethesda Res. Lab. (Rockville, MD). The systems as supplied had been made dependent on exogenous mRNA by pretreatment with micrococcal nuclease. Reticulocyte reaction mixtures (30 μl) contained: lysate (A_{260} = 194), 10 μl; globin mRNA, 13 μg/ml; [^{3}H]leucine, 2.5 μCi; $MgCl_2$, 1.2 mM; EDTA, 17 μM; KCl, 48 mM; potassium acetate, 87 mM; DTT, 0.17 mM; hemin, 8.3 μM; $CaCl_2$, 0.33 mM; EGTA, 0.67 mM; NaCl, 40 mM; Hepes, 25 mM; Tris, 6.7 mM; creatine kinase, 17 μg/ml; creatine phosphate, 33 mM; amino acids minus leucine (170 μM each) and varying amounts

of toxin. Wheat germ reaction mixtures (30 μl) contained: wheat germ extract (A_{260} = 80), 10 μl; globin mRNA, 13 μg/ml; [^{3}H]leucine, 0.20 μCi; Hepes, 26 mM; magnesium acetate, 2.0 mM; KCl, 33 mM; potassium acetate, 63 mM; 2-ME, 1.3 mM; ATP, 1.2 mM; GTP, 0.10 mM; creatine phosphate, 5.5 mM; creatine kinase, 0.20 mg/ml; spermidine phosphate, 80 μM; amino acids minus leucine (50 μM each) and varying amounts of toxin. Before use, [4,5-^{3}H]leucine (Amersham) was lyophilized, redissolved in distilled H_2O and adjusted to 14 Ci/mmol. Incorporation into trichloroacetic acid-precipitable material was assayed by spotting portions (5 μl) on paper discs (Schleicher and Schuell no. 740E), following the method in [16]. Washed discs were incubated for 20 min with 0.5 ml NCS (Amersham), then assayed for radioactivity with Liquifluor scintillation solution (New England Nuclear).

Protein concentration was determined by the method in [17]. Polysome profiles were determined by rate zonal centrifugation in 15–30% sucrose gradients above a 50% sucrose cushion at 40 000 rev./min for 2.75 h using a Beckman SW 41 rotor [18]. ADP-ribosylation activity was assayed by incorporation of radioactivity from nicotinamide [U-^{14}C]adenine dinucleotide (270 mCi/mmol), using EF-2 from wheat germ homogenates as acceptor [11]. EF-2 was obtained as the 30–50% saturated ammonium sulfate fraction.

3. Results

Purified Shiga toxin inhibited [^{3}H]leucine incorporation in globin mRNA-dependent protein synthetic mixtures from either rabbit reticulocyte lysates or wheat germ extracts only at relatively high toxin protein concentrations. 50% inhibition occurred at ~6 μg toxin/ml. The preparation was pretreated with various reagents to determine whether toxin existed as an inactive proenzyme. When toxin was pretreated with 8 M urea plus 10 mM DTT, a 70-fold enhancement of the ability to inhibit cell-free protein synthesis in rabbit reticulocyte lysates was observed (fig.1). Toxin at ~80 ng/ml gave 50% inhibition. In the cell-free system, incubation of toxin with 10 mM DTT alone provided ~20-fold activation over untreated toxin. Treatment with 8 M urea resulted in a smaller degree of activation (table 1). Proteolytic treatment followed by disulfide reduction also enhanced the inhibitory activity of purified toxin. Pretreatment with Triton X-100, sodium deoxycholate or low pH buffer did not enhance activity to the same degree. Inhibitory activity was completely destroyed by heating to 100°C for 15 min.

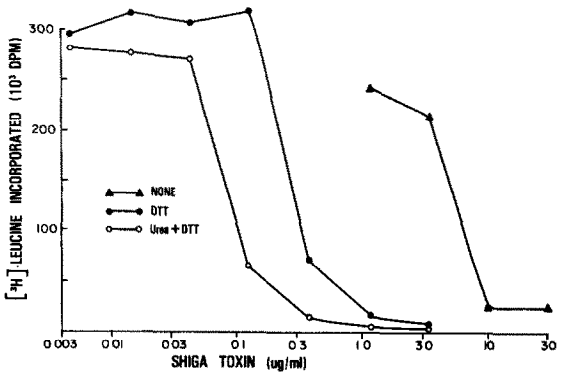

Fig.1. Effect of reduction and denaturation of Shiga toxin on inhibition of reticulocyte cell-free protein synthesis. Portions (10 μl) of Shiga toxin (0.55 mg/ml) were transferred to conical plastic tubes containing either 2 μl 60 mM DTT or 7.5 mg urea plus DTT. The vials were agitated and then incubated at 37°C for 60 min. Samples were diluted to 270 μl, then serially 3-fold, with cold 0.02 M Tris–HCl (pH 8), 0.01 M NaCl. Untreated toxin remained at 0°C and was diluted serially 3-fold with the same buffer. Toxin dilutions (5 μl) were added to the complete synthetic reaction mixture at 0°C and the vials were incubated at 30°C for 1 h. Concentrations shown are those in the synthetic reaction mixture.

Incubation of toxin with 8 M urea, 10 mM DTT resulted in a concomitant loss of cytotoxicity. The 50% cytotoxic dose (CD_{50}) for the purified toxin was 50 pg/ml. Treated toxin had a CD_{50} of 500 pg/ml, a decrease in cytotoxicity of 90%. The effect of the other reagents on cytotoxic activity was not tested.

To investigate the mechanism of inhibition by the activated toxin, the effects on kinetics of [^{3}H]leucine incorporation and on the polysome profile after inhibition were examined. Fig.2 shows the time course of inhibition. Before toxin addition, incorporation of [^{3}H]leucine proceeded in a linear fashion. Addition of toxin (~18-fold more concentrated than 50% inhibition level) resulted in an immediate inhibition of protein synthesis, whereas the control mixture continued active incorporation for at least 30 min more. Sucrose density gradient centrifugation of the wheat germ protein synthetic mixture was performed to examine the effect of toxin on nascent peptide synthesis (fig.3). Incorporation of [^{3}H]leucine

Table 1
Effect of toxin pretreatment of inhibition of protein synthesis

Treatment at 37°C[a]	Incorporation of [^{3}H]leucine (10^3dpm)			Inhibition[b] (%)
Shiga toxin (μg/ml):	0.0	1.3	3.4	
Buffer	304	–	105	67
Urea 8 M	–	44.8	–	86
DTT 10 mM	339	13.4	11.2	96
Urea 8 M/DTT 10 mM	302	4.9	6.5	98
Trypsin (1 μg/ml); 2-ME (0.1%)[c]	352	–	21.1	93
Chymotrypsin (1 μg/ml); 2-ME (0.1%)[c]	308	–	14.1	95
Triton X-100 (0.1%)	302	–	68.7	78
Sodium deoxycholate (0.1%)	307	–	57.4	82
Acetic acid 0.1 M (pH 3)	324	–	94.4	70
100°C, 15 min	320	–	325	0

[a] Reaction mixtures were incubated at 37°C for 60 min, then diluted 27-fold with 0.020 M Tris–HCl (pH 8.0), 0.10 M NaCl. Aliquots (5 μl) were assayed with rabbit reticulocyte lysate

[b] Calculated from mean of control values = 318 000 ± 18 000 dpm as 0% inhibition

[c] 2-ME was added after 37°C incubation, then phenylmethylsulfonyl fluoride (0.02 μg/ml) was added before the final dilution to inhibit further proteolysis

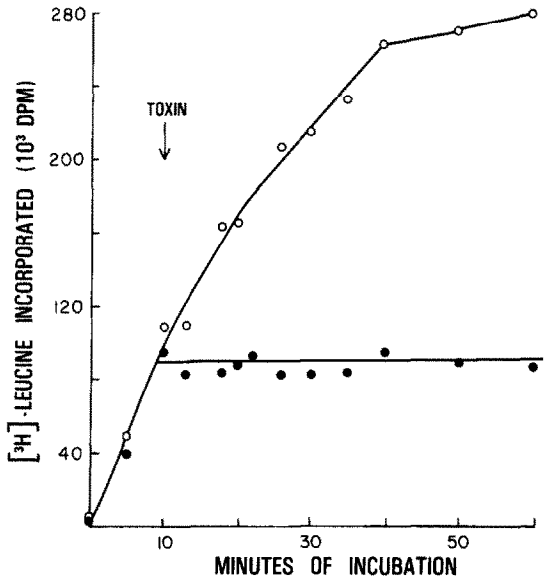

Fig.2. Effect of activated Shiga toxin on incorporation of [^{3}H]leucine by rabbit reticulocyte lysates. Toxin was incubated in 8 M urea, 10 mM DTT, for 60 min at 37°C, cooled and diluted 27-fold. Complete synthetic reaction mixtures (90 μl) were brought to 30°C. At 10 min toxin (•) or buffer (○) were added. At the indicated times aliquots (5 μl) were transferred to paper discs, which were immediately plunged into 10% trichloroacetic acid. Toxin was 1.1 μg/ml.

was allowed to proceed for 10 min, toxin was added, and the mixture was incubated for an additional 10 min. The mixture (250 μl) was then subjected to centrifugation in a sucrose gradient and subsequent fractionation. Both the toxin-treated mixture and the control sample contained numerous polysomal species with attached labeled peptidyl-tRNA. Control polysomes contained more labeled nascent polypeptide, which resulted from the longer period of linear incorporation of amino acids in the uninhibited mixture. Reaction mixture lacking mRNA had no significant incorporation.

Diphtheria toxin and *P. aeruginosa* exotoxin A are well known to inhibit peptidyl elongation by ADP-ribosylation of EF-2 [10]. Activated Shiga toxin was assayed to determine if inhibition occurred by this mechanism (table 2). No incorporation of [^{14}C]ADP-ribose was observed with either untreated toxin or toxin treated with DTT, urea plus DTT, SDS plus DTT, or trypsin. Fragment A of diphtheria toxin, which served as a positive control, displayed ADP-ribosyl transferase activity after all pretreatment procedures.

4. Discussion

Shiga toxin apparently is synthesized in the bacterial cell as a zymogen, requiring modification before

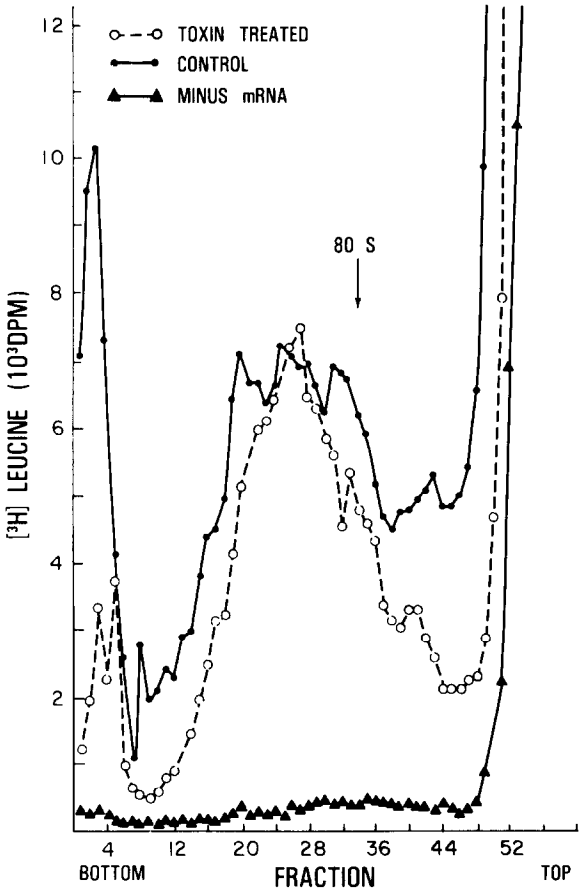

Fig.3. Gradient centrifugation pattern of wheat germ extract incorporation mixture. Toxin was activated as in fig.2. Reaction mixtures containing 0.30 mCi/ml [^{3}H]leucine (140 Ci/mmol) were incubated 10 min at 30°C. Activated toxin (1.1 μg/ml) or buffer was added and incubation continued for an additional 10 min. After centrifugation, fractions (0.2 ml) were collected and assayed for radioactivity (25 μl) and A_{260}. Profiles were aligned from the 80 S ribosomal absorbance peaks.

acquiring intracellular activity. Pretreatment of the purified toxin enhanced activity such that inhibition of protein synthesis occurred in a catalytic fashion. Using a value of 4 mg ribosomes/ml packed reticulocytes (B. Hardesty, personal communication), we calculate a simple ratio of at least 100 ribosomes/activated toxin molecule at the 50% inhibitory concentration (fig.1). These studies confirm our observation that the primary cytotoxic effect of Shiga toxin is an enzymatic inhibition of protein synthesis [7]. Loss of cytotoxicity occurred concomitantly with increased cell-free inhibition. Since Shiga toxin is

Table 2
ADP-ribosylation activity after toxin pretreatment

Treatment[a]	[^{14}C]ADP-ribose incorporated	
	Shiga toxin[b]	Diphtheria toxin fragment A[c]
	(cpm/reaction mixture)[d]	
None	160	5420
DTT 80 mM	120	4870
Urea 7.5 M/DTT 80 mM	140	1770
0.1% SDS/DTT 80 mM	90	3470
Trypsin (1 μg/ml)	20	5590

[a] Samples were incubated 15 min at 23°C, diluted 3-fold with 0.1 M Tris (pH 8) containing 1 mg/ml bovine serum albumin. Soybean trypsin inhibitor (62 μg/ml) was added to the trypsin sample. Portions were assayed for ADP-ribosylation activity.
[b] Final conc., 7.0 μg/ml
[c] Final conc., 0.013 μg/ml
[d] Average of duplicate determinations, minus background radioactivity

an oligomeric protein [5,15], inhibition of protein synthesis and cell surface binding may be specific properties of separate subunits. The mechanism of activation is not clear from these studies. Either a conformational process or proteolytic cleavage may be sufficient to convert the protein to its active state. In either case, disulfide reduction appears critical to the activation process.

These studies have not demonstrated that inhibition by activated toxin is a direct consequence of inactivation of the ribosome. Nonetheless, such inhibition by Shiga toxin does not result from inactivation of other components of the translation system. No inhibition of aminoacylation of tRNA in a rat liver-derived system was observed [8]. Moreover, in a poly(U)-directed bacterial mixture, pre-incubation of poly(U), tRNA fraction, or S-105 fraction did not inhibit incorporation. Only pre-incubation of the ribosomal fraction inactivated the mixture [9]. Our data with activated toxin are consistent with a direct ribosomal mechanism since no lag period was observed after toxin addition, and intact polysomes were preserved after complete inhibition.

Inhibition of peptidyl elongation appears to be the primary mechanism of inhibition of protein synthesis. Inhibition occurred immediately after toxin addition suggesting an instantaneous cessation of protein synthesis. The polysomal profile was preserved

in the toxin-treated sample, demonstrating that Shiga toxin had prevented the completion and liberation of already initiated peptide chains. These data do not elucidate the actual reaction mechanism of toxin action. Three possible mechanisms of inhibition have been observed with other toxic proteins inhibiting cell-free protein synthesis: ADP-ribosylation of EF-2 by diphtheria toxin and *P. aeruginosa* exotoxin A- [10], inactivation of the 60 S subunit by abrin and ricin [13] or nicking of 16 S rRNA by colicin E3 [14]. ADP-ribosylation of EF-2 does not appear to be the mechanism, which agrees with the observation that addition of exogenous NAD^+ does not enhance the inhibition of cell-free protein synthesis [8]. Inhibition of protein synthesis by ADP-ribosylation of some other component of the translation system can not be ruled out on the basis of these data. The mechanism of toxin activation as well as the mechanism of ribosomal inhibition are under investigation.

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