THE PREPARATION OF STABLE, BIOLOGICALLY ACTIVE B FRAGMENT OF DIPHTHERIA TOXIN

R.D. Burgoyne, J. Wolstenholme and J. Stephen

Department of Microbiology, University of Birmingham, P.O. Box 363, B15 2TT England.

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SUMMARY. A 'nicked' preparation of diphtheria toxin was dissociated by treatment with dithiothreitol, and treated with iodoacetic acid. A and B fragments were purified in biologically active states by chromatography on Sephadex G75 Superfine in the presence of 6M guanidine HCl. The isolated B fragment, when mixed with isolated A fragment reconstituted the toxicity of A to whole cells. The B fragment was also used to induce the intracytoplasmic uptake of an unrelated protein.

Diphtheria toxin consists of two polypeptide fragments A and B (of molecular weights 24,000 and 39,000 respectively) which can be separated after 'nicking' the toxin with trypsin and reduction with thiol reagents (1,2). The A fragment is highly stable, can be easily purified in an active form, and is not toxic to whole cells though it has been demonstrated to be active in inhibition of protein synthesis in a cell free system (1,2). Using mutant forms of toxin, B fragment has been shown to interact with cells and facilitate the intracytoplasmic uptake of the enzymically active A fragment (3). Thus far B fragment has not been purified in a demonstrably biologically active state due to its inherent instability after purification.

This paper reports the purification of the B fragment of diphtheria toxin in a sufficiently native form to allow reconstitution of active toxin when mixed with A fragment and induction of uptake of an unrelated intracellularly acting toxic protein.

MATERIALS AND METHODS. Diphtheria toxin, batch No. JG 769/2/10, containing 0.01% thiomersalate was a gift from Dr. R.O. Thomson, Wellcome Research Laboratories, Beckenham, Kent, England. The thiomersalate was removed on a

Sephadex G25 column eluted with 0.4M Tris HC1 pH 8.0 buffer. The toxin (6 mg/ml) which was almost completely 'nicked' was reductively cleaved A and B fragments, by addition of dithiothreitol to a concentration of 23mM at 37°C for 3 h in the presence of 6M guanidine HC1. Cleaved toxin was treated with iodoacetic acid (final concentration 65 mM) at 20°C in the dark for 1 h. The mixture was then fractionated at room temperature on a Sephadex G75 (Superfine) column (45 cm x 3.5 cm) in 0.4M Tris HC1 buffer pH 8.0 containing 6M guanidine HC1 at a flow rate of 10 ml/h.

Protein concentration was assessed by determination of total protein nitrogen after extensive dialysis of the sample to remove guanidine HC1.

Polyacrylamide gel electrophoresis on 10% gel in the presence of 0.1% SDS was carried out according to Weber and Osborn (4); sample buffer did not contain thiol reagent unless indicated.

Toxicity testing was carried out on dispersed monolayers of HeLa cells in wells of microtitre plates (NUNC N1480). Test solutions were dialysed against growth medium (MEM) for 24 h, millipore filtered, then added to cells for 15 min. At the end of this exposure period test solutions were removed, the cells washed twice with MEM, and subsequently incubated for 24 h. Cells were then counted and cell survival expressed as a percentage of MEM treated controls. With this system the Tissue Culture Lethal Dose 50 of diphtheria toxin for HeLa cells was found to be 0.135 µg/ml (2.1×10^{-9}M).

Partially purified vaccinia virus specific toxic protein (5) was also used in uptake studies with B fragment.

Solutions of B and A fragments (or B and vaccinia toxic protein) in 6M guanidine HC1 were mixed and the guanidine HC1 removed by dialysis for 24 h against MEM with several changes of medium.

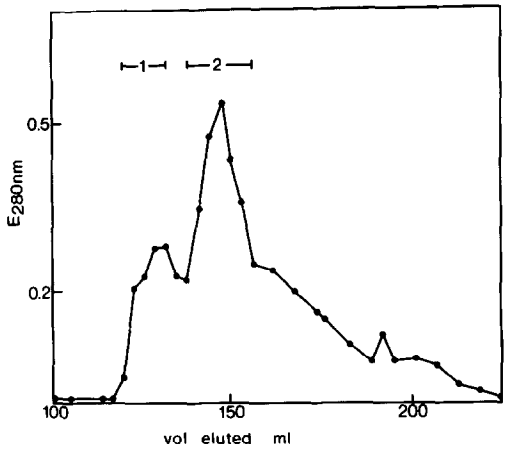

Fig. 1. Fractionation of cleaved toxin on Sephadex G75 Superfine. 2 ml of mixture (6 mg protein/ml) were loaded onto a column (45 cm x 3.5 cm) and eluted with 0.4M Tris HC1 pH 8.0 buffer containing 6M guanidine HC1 at room temperature, at a flow rate of 10 ml/h and 3 ml fractions collected. The fractions were pooled as shown above.

RESULTS AND DISCUSSION

Purification of the B fragment of diphtheria toxin. Chromatography of treated toxin on a Sephadex G75 Superfine column (Fig. 1) gave yields of A and B fragments of 40 and 15% respectively of total applied protein. Though a relatively low yield of B fragment was obtained, this was not grossly contaminated by intact toxin or A fragment as seen by SDS-polyacrylamide gel electrophoresis (Fig. 2). The B fragment was found to be stable on prolonged storage in 6M guanidine HCl at -20°C. After dialysis for 24 h against neutral buffer at 4°C to remove guanidine HCl, B fragment remained in solution and was stable at room temperature for several hours.

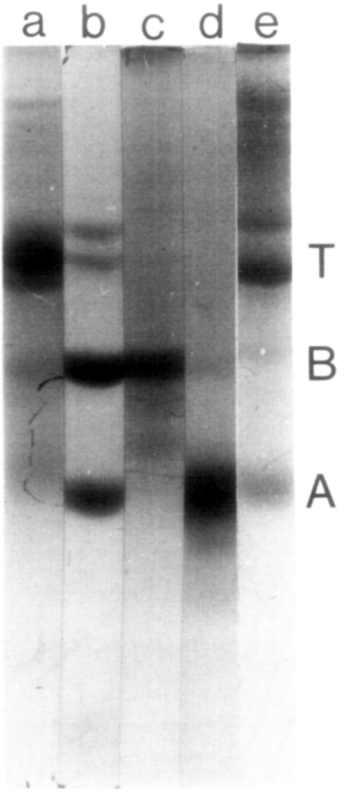

Fig. 2. SDS-polyacrylamide gel electrophoresis of a) untreated toxin, b) toxin in the presence of 2-mercaptoethanol, c) fraction 1 (B fragment) from G75 column, d) fraction 2 (A fragment) from G75 column and e) dialysed mixture of A and B fragment.

Demonstration of the biological activity of B fragment by reconstitution of active, intact toxin. Examination of dialysed B and A mixtures on SDS-polyacrylamide gels indicated the formation of a product corresponding to intact toxin as well as higher molecular weight material, possibly dimers or trimers of toxin as have been seen by other workers (6,7). Polymeric material could also be detected in control untreated preparations of toxin. The carboxymethylation treatment was obviously not successful in completely blocking free -SH groups on the polypeptides, under the conditions used.

A and B fragments were not toxic on their own at concentrations up to $3x10^{-6}$M and $4.4x10^{-7}$ respectively. A and B fragments each at non toxic concentrations were mixed, dialysed and the mixture tested for toxicity (Table 1). Purified B fragment was still in a sufficiently native form in solution to reconstitute toxicity of A, although at a level significantly lower than would be the case for the same number of A fragments present in native toxin. This probably reflects the number of successful A and B molecules formed during a process of random combinations.

Induction of uptake by B fragment of an unrelated protein. Work in this laboratory (5) has lead to the detection of a vaccinia virus-specific protein in extracts of infected HeLa cells which though non toxic alone to healthy cells, is toxic in the presence of uptake inducers such as hypertonic salt solutions. Attempts were made to induce the uptake of this acidic protein under more physiological conditions using the B fragment of diphtheria toxin.

Experiments were carried out involving mixing of B fragment at non toxic concentrations with the vaccinia protein in 6M guanidine HCl followed by dialysis against MEM to allow interaction of the two molecules. The dialysed mixture was demonstrated to be toxic to HeLa cells (Table 1), indicating successful uptake of the vaccinia protein. The latter did not kill as many cells with B fragment as did the A fragment (at the higher

Table 1

TOXICITY OF DIALYSED MIXTURES OF B AND A FRAGMENTS, AND B FRAGMENT AND VACCINIA TOXIC PROTEIN (VT) TO HELA CELLS

Test Mixture			% of HeLa cells killed[2]
Diphtheria B (M)	Diphtheria A (M)	VT (μg/ml)[1]	
4.4×10^{-8}	-	-	0
-	3.8×10^{-7}	-	0
-	-	440	0
4.4×10^{-8}	3.8×10^{-7}	-	35
4.4×10^{-8}	-	440	N.D.[3]
1.1×10^{-7}	-	-	0
1.1×10^{-7}	3.8×10^{-7}	-	24
1.1×10^{-7}	-	440	35
4.4×10^{-7}	-	-	0
4.4×10^{-7}	3.8×10^{-7}	-	61
4.4×10^{-7}	-	440	25

1. This preparation was only partially purified; 440 μg/ml protein was used in each test and mixed with B as indicated.

2. Calculated as difference in survivors between 'toxin-treated' and MEM control cells.

3. N.D., not done

concentration of B fragment) probably reflecting a lesser interaction between the heterologous molecules. A and B fragments are possibly reassembled to give some biologically active intact toxin, whereas B fragment and vaccinia protein are probably only held together by non covalent interaction.

Alternatively uptake induction could be occurring as a result of a non specific effect of B fragment on the cell membrane.

It may now be possible to investigate more closely the interaction of B fragment (prepared in this manner) with cell membranes. It may also be possible to extend the observations reported here to include the induced intracytoplasmic uptake of other unrelated macromolecules.

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