Association between diphtheria toxin A- and B-fragment and their fusion proteins

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Natural diphtheria toxin is synthesized as a single polypeptide chain that is activated by cleavage into an A- and a B-fragment, which are linked by a disulphide bond. In the present work the ability of independently translated A- and B-fragments to associate was investigated. Low amounts of A- and B-fragments synthesized *in vitro* were mixed under conditions that allowed formation of a disulphide bridge between the fragments. Under these conditions toxin was reconstituted in close to 100 % yield and found to be as toxic to Vero cells as natural diphtheria toxin. Efficient association between the A- and B-fragment was dependent on the formation of a disulphide bridge. Reconstituted toxin obtained from one [³⁵S]methionine-labelled fragment and one unlabelled fragment proved useful in translocation studies. Addition of a number of different polypeptides to the N- and C-termini of either fragment did not, in most cases, prevent reconstitution. The ready reconstitution allows easy manipulations with the toxin to form targeted molecules and to develop diphtheria toxin as a vector for translocation of peptides to the cytosol. The fact that the reconstituted toxin does not need to be nicked with proteinases to be active allows experimentation with proteinase-sensitive constructs.

INTRODUCTION

A number of plant and bacterial toxins inhibit protein synthesis by acting on intracellular targets (Olsnes & Sandvig, 1985). Owing to their very high potency, these toxins are often used to arm antibodies against epitopes on tumour cells in attempts to form cancer-specific drugs (immunotoxins, see Frankel, 1988). Fusion proteins between toxins and hormones or growth factors are also promising candidates for specific cytotoxic agents (Kelley *et al.*, 1988; Nichols *et al.*, 1990). Manipulations of the toxin conjugates to optimize the action of fusion proteins is central to this work.

Diphtheria toxin is one of the toxins most commonly used for these purposes. This toxin is secreted by *Corynebacterium diphtheriae* as a single polypeptide chain of 58 kDa, which is proteolytically nicked into two disulphide-linked fragments, A (21 kDa) and B (37 kDa) (Drazin *et al.*, 1971). The A-fragment is an enzyme that inhibits protein synthesis, whereas the B-fragment is responsible for transporting the A-fragment to the cytosol of target cells.

Studies of diphtheria toxin fusion proteins have, however, been hampered by the fact that active diphtheria toxin has been considered too dangerous to be cloned and expressed in *E. coli*. Genes for inactive mutants of the toxins have been cloned (Greenfield *et al.*, 1983; Wilson *et al.*, 1990), and recently we presented a method to express active diphtheria toxin in a cell-free system by ligation *in vitro* of DNA fragments corresponding to the A- and the B-fragment, which are non-toxic by themselves (Olsnes *et al.*, 1989). The reconstituted gene was then transcribed and translated in a cell-free system. Amplification of the ligated DNA by the polymerase chain reaction yielded a large supply of the reconstituted gene.

A major problem with this method of making chimeric toxin is that after translation the toxin must be activated by proteolytic cleavage between the A- and the B-fragment. Such cleavage is easily carried out with natural toxin, since the region linking the

two different parts of the toxins is approx. 100-fold more sensitive to trypsin than the remaining part of the toxin. However, when fusion proteins are made where the toxin is linked to peptides or proteins, the proteinase treatment often cleaves the added material from the toxin (Stenmark *et al.*, 1991). To overcome these problems, we have taken advantage of the fact that separate A- and B-fragments may reassociate on removal of the reducing agent (Uchida *et al.*, 1972). We first investigated the efficiency of forming toxin from low concentrations of separately translated A- and B-fragments thus avoiding the nicking step. Furthermore, we have studied to what extent various peptides and proteins linked to the N- and C-termini of the toxin fragments interfere with the reassociation.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli strain TG1 was used as the host during the site-directed mutagenesis step, and *E. coli* strains DH5 α and JM105 were used in the cloning procedures.

Cell culture

Vero cells were propagated under standard conditions and seeded into 12- or 24-well disposable plates 2 days before the experiments, at a density of 10⁵ and 5 $\times$ 10⁴ cells/well respectively.

Buffers

The following buffers were used: Hepes medium, Eagle's minimum essential medium buffered with 20 mM-Hepes to the pH indicated; phosphate-buffered saline (PBS), 140 mM-NaCl/10 mM-NaH₂PO₄, pH 7.4; Mes/gluconate buffer, 140 mM-NaCl/5 mM-Mes/5 mM-sodium gluconate, pH 4.8.

Preparation of fragment A from natural diphtheria toxin

Diphtheria toxin (Connaught Laboratories, Willowdale, Ont, Canada) was purified as described by Sandvig & Olsnes (1981).

Abbreviations used: PBS, phosphate-buffered saline; Tos-Phe-CH₂Cl, *N*-tosyl-L-phenylalanylchloromethane ('TPCK'); DHFR, dihydrofolate reductase.

unlabelled methionine. The radioactive translation products were analysed by SDS/PAGE and fluorography. Translation in the absence of specific mRNA yielded only small amounts of a protein of 16 kDa. The isolated gel bands were incubated with Opti-Fluor (Amersham) for 30 min, and the radioactivity was measured in a Packard liquid-scintillation counter. The concentration (c) of translated protein was calculated according to the following formula:

$$c = \frac{c_1}{c_2} \times \frac{r}{aVnf}$$

where c_1 is the concentration of unlabelled methionine (which is the sum of the added methionine and the $5 \mu\text{M}$ present in the lysate), c_2 is the concentration of [^{35}S]methionine, r is the measured radioactivity, a is the specific radioactivity of [^{35}S]methionine, n is the number of methionine residues according to the amino acid sequence, V is the volume of lysate analysed by SDS/PAGE, and f is the counting efficiency of ^{35}S -labelled proteins in gel slices (this value was 85% in the present experiments).

Association between A- and B-fragments

Reticulocyte lysate systems containing toxin fragments formed in the absence or presence of [^{35}S]methionine were mixed in approximately equimolar amounts (about 1 nM of each fragment) and dialysed overnight at 4°C in Eppendorf tube caps covered with dialysis membrane (cut-off 3500) against a > 1000 -fold excess of PBS and then for 4 h at 4°C against Hepes medium. There was free access to atmospheric oxygen. Control experiments showed that the disulphide formation was complete after 5 h. There was no measurable pH change in the PBS and Hepes medium which remained at pH 7.4.

RESULTS

Toxin reconstitution

[^{35}S]Methionine-labelled A- and B-fragments were formed in a cell-free system from rabbit reticulocyte lysate as described in the Materials and methods section and mixed in approximately equimolar amounts. The mixture was extensively dialysed against PBS to remove reducing agents and unincorporated [^{35}S]methionine and then analysed by SDS/PAGE. As shown in Fig. 2, most of the A- and B-fragments (lane 1 and 2) associated during the dialysis to form whole toxin (lane 6). On treatment with 2-mercaptoethanol to reduce the interfragment disulphide, the free A- and B-fragments reappeared (lane 3). It should be noted that small amounts of additional bands appeared after dialysis to form reconstituted toxin (lane 6). These bands also appeared when A-fragment (lane 4) or B-fragment (lane 5) were dialysed alone. Their size suggests that they represent disulphide formation with globin chains (16 kDa) which are abundant in the reticulocyte lysate.

To test if the protein formed was toxic, the experiment was repeated with unlabelled A- and B-fragments. The separate fragments as well as the reconstituted toxin were added to Vero cells (Middlebrook *et al.*, 1978) and their ability to inhibit protein synthesis was measured. The data in Fig. 3 show that the reconstituted toxin was as toxic as natural diphtheria toxin, whereas the separated fragments did not induce any measurable toxic effect.

From the data in Fig. 2, lane 6, it appears that the association of the two fragments progressed until the fragment present in the lowest concentration (in this case the A-fragment) was completely consumed. To test this in more detail, we took advantage of the fact that diphtheria toxin forms cation-selective channels in cells

on exposure to low pH (Sandvig & Olsnes, 1988) and that the free B-fragment is approx. 100 times more efficient in this respect than the whole toxin (Stenmark *et al.*, 1989). Therefore, if all B-fragment is consumed in the presence of excess A-fragment, the ability of the mixture to form cation-channels should be strongly reduced. We therefore mixed B-fragment synthesized *in vitro* with excess A-fragment purified from reduced natural diphtheria toxin. After dialysis, the mixture was added to Vero cells and the influx of $^{22}\text{Na}^+$ at low pH was measured. The data in Fig. 4 show that the ability of the dialysed mixture to permeabilize the cells to $^{22}\text{Na}^+$ was reduced to approximately one-thirtieth of that

Fig. 2. Association of A- and B-fragments synthesized in a cell-free system

pBD-30 and pBD-23 were transcribed and the mRNA was translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine to yield fragment A and fragment B respectively. The translation systems, both separate and mixed, were dialysed overnight against Hepes medium and analysed by SDS/PAGE and fluorography. Lanes 1–3, samples treated with 5% 2-mercaptoethanol before electrophoresis. Lane 1, fragment A alone; lane 2, fragment B alone; lane 3, dialysed mixture of A- and B-fragments. Lanes 4–6, non-reducing conditions. Lane 4, dialysed A-fragment; lane 5, dialysed B-fragment; lane 6, dialysed mixture of A- and B-fragments.

Fig. 3. Toxic effect of reconstituted diphtheria toxin

pBD-30 and pBD-23 were transcribed and translated in the presence of unlabelled methionine as described in the Materials and methods section. Part of the translation products was dialysed separately, whereas the rest were mixed in equal amounts and dialysed overnight against Hepes medium. Increasing amounts of the dialysed samples were added to Vero cells growing in 24-well microtitre plates, and incubated overnight. The next day the rate of protein synthesis was measured as the ability of the cells to incorporate [^3H]leucine during 30 min. $\square$, Fragment A alone; $\triangle$, fragment B alone, $\circ$, fragment A and B dialysed together; $\bullet$, natural diphtheria toxin.

Fig. 4. Ability of free and reassociated B-fragment to form cation channels in Vero cells

B-fragment and B-36 were formed *in vitro* as in Fig. 3. They were dialysed either alone or together with a 10-fold excess of A-fragment isolated from natural diphtheria toxin and then added to Vero cells in 24-well disposable trays. After 20 min at 24 °C, the medium was removed and Mes/gluconate buffer, adjusted to pH 4.8 and containing 2 μ Ci of $^{22}\text{Na}^+$ /ml, 1 mM-amiloride and 50 μ M-ouabain, was added. After 8 min at 37 °C the cells were washed three times with ice-cold PBS and the radioactivity associated with the cells was extracted with 200 μ l of 5% (v/v) trichloroacetic acid and measured. Δ , B-fragment alone; $\blacktriangle$, B-fragment dialysed together with A-fragment; $\bullet$, B-36 alone; $\circ$, B-36 dialysed together with A-fragment.

obtained with B-fragment alone, indicating that essentially all the B-fragment had formed heterodimers with the A-fragment. In contrast, B-36, a truncated B-fragment lacking Cys-201, retained the high channel-forming ability in the presence of A-fragment.

Membrane translocation of reconstituted toxin

By reconstituting diphtheria toxin from separate fragments, toxin can be made which is labelled exclusively in one of its constituent fragments. This facilitates the interpretation of translocation experiments. In Fig. 5 toxin is shown reconstituted from labelled A-fragment and unlabelled B-fragment (lane 3) and labelled B-fragment and unlabelled A-fragment (lane 4). As expected, in both cases only one labelled fragment appeared on reduction (lanes 1 and 2).

The reconstituted toxins were bound to cells in the same way as ^{125}I -labelled natural toxin. In the presence of excess unlabelled toxin very little binding of labelled toxin occurred indicating that in each case the binding was specific (results not shown).

When ^{125}I -labelled toxin is bound to cells and the cells are then exposed to low pH, part of the bound toxin is translocated to the cytosol (Moskaug *et al.*, 1988). After treatment with Pronase to remove material exposed at the cell surface, two protected fragments are obtained migrating in positions corresponding to 21 kDa and 25 kDa (Fig. 6, lane 4). The most rapidly migrating band corresponds to A-fragment translocated to the cytosol whereas the 25 kDa fragment is derived from the B-fragment and

Fig. 5. Reconstitution of diphtheria toxin from one labelled and one unlabelled fragment

Labelled A-fragment and unlabelled B-fragment and vice versa were mixed and dialysed overnight and then analysed by SDS/PAGE with (lanes 1 and 2) and without (lanes 3 and 4) pretreatment with 2-mercaptoethanol. The gels were analysed by fluorography. Lanes 1 and 3, [^{35}S]methionine-labelled fragment A + unlabelled fragment B; lanes 2 and 4, unlabelled fragment A + [^{35}S]methionine-labelled fragment B.

Fig. 6. Translocation to the cytosol of reconstituted toxin

^{125}I -labelled natural toxin and toxin synthesized *in vitro* labelled in both fragments or in only one fragment as described in Fig. 5 were added to Vero cells which were kept for 20 min at 24 °C, then washed and exposed to Mes/gluconate buffer, pH 4.8, for 2 min at 37 °C. Subsequently, the cells were treated with 3 mg of Pronase E/ml at 37 °C for 5 min, washed with Hepes medium containing 1 mM-*N*-ethylmaleimide and 1 mM-phenylmethanesulphonyl fluoride and analysed by non-reducing SDS/PAGE and fluorography. Lane 1, reconstituted toxin where both fragments were labelled with [^{35}S]methionine; lane 2, reconstituted toxin with [^{35}S]methionine-labelled A-fragment; lane 3, reconstituted toxin with [^{35}S]methionine-labelled B-fragment; lane 4, ^{125}I -labelled toxin alone.

appears to be inserted into the membrane (Moskaug *et al.*, 1987). As shown in Fig. 6, only the 21 kDa band was obtained with toxin labelled selectively in the A-fragment (lane 2) and only the 25 kDa band was obtained when the labelling was in the B-fragment (lane 3). When both the A- and B-fragment were labelled, both the 25 kDa band and the 21 kDa band were obtained (lane 1).

Importance of interfragment disulphide bridge

Although the A- and the B-fragment of the toxin are normally linked by a disulphide bond, the two fragments appear to be also

Fig. 7. Requirement of an interfragment disulphide bridge for reconstitution of toxin

Mixtures of lysates containing B3-A and B-36 (lanes 1, 3 and 5) or B3-A and whole B-fragment (lanes 2, 4 and 6) were either analysed directly by reducing SDS/PAGE (lanes 1 and 2) or after immunoprecipitation with anti-B3 serum and protein A-Sepharose (lanes 3 and 4). Protein A-Sepharose not preincubated with anti-B3 serum was incubated with samples of the lysates as controls (lanes 5 and 6). The Figure represents fluorograms of the gel.

held together by weak interactions (Gill & Dinius, 1971). To test if the two fragments associate to form a stable heterodimer even under conditions where the cysteine residue of the B-fragment involved in the interfragment disulphide is lacking, we mixed such a modified B-fragment (B-36) with A-fragment carrying a tetradecapeptide, B3, linked to its *N*-terminus (Fig. 7, lane 1). After dialysis, the A-fragment was immunoprecipitated with an antiserum against the B3 peptide, and the precipitate was analysed for the presence of B-fragment. The results showed that hardly any B-36 was present in the immunoprecipitate (lane 3). On the other hand, when B-fragment containing Cys-201 was used, anti-B3 serum precipitated the B-fragment together with the A-fragment (lane 4). The data therefore indicate that the interfragment disulphide bridge is necessary to form a stable complex.

A short sequence (Ser-194-Val-Gly-Ser-Ser-Leu-Ser-Cys-Ile-202) present at the *N*-terminus of natural B-fragment is deleted in B-36 and replaced by Met-Ala-Leu. It is, however, unlikely that this sequence is important for reassociation of the A- and the B-fragment since B-36C (McGill *et al.*, 1989), where the sequence Ser-194 to Ser-200 is replaced by an unrelated sequence (Met-Ala-Pro-Trp-Pro), and where Cys-201 is retained, was found to reassociate quantitatively with A-fragment (results not shown). The results in Fig. 7 are consistent with the finding in Fig. 4 that B-36 retains its high channel-forming ability when mixed with A-fragment, whereas the channel-forming ability of the B-fragment is greatly decreased after dialysis together with A-fragment.

Reconstitution of toxin containing additional peptide material

To target diphtheria toxin to particular cell types, binding moieties providing selectivity may be linked to the C-terminus of the toxin (Kelley *et al.*, 1988). We are now investigating the possibility of developing diphtheria toxin as a vector to bring peptides and proteins into the cytosol (Stenmark *et al.*, 1991). As part of this investigation we tested whether toxin can be

reconstituted with peptides and proteins linked in front of and behind the toxin fragments. The results are demonstrated in Fig. 8. In each of the ten panels (Fig. 8a–Fig. 8j) is shown a reconstitution experiment where the sample is analysed by SDS/PAGE under reducing (lanes labelled R) and non-reducing conditions (labelled N). It should be noted that the different panels represent independent experiments and that the migration rates should only be compared within each panel. The positions of reconstituted toxin and the constituent peptides are indicated in each case.

First we made a number of constructs with *Staphylococcal* protein A, which binds to the Fc part of immunoglobulins (Löwenadler *et al.*, 1987). The data in Fig. 8 show that A-fragment alone (Fig. 8a) as well as toxin A-fragments with a synthetic duplicated B-domain of protein A (below referred to as ZZ) as its *N*-terminus (Fig. 8f) and C-terminus (Fig. 8g) were able to associate with B-fragment. B-fragment with ZZ linked to the C-terminus (Fig. 8h) and at the *N*-terminus (Fig. 8i) were able to associate with A-fragment. It therefore appears that ZZ located at either end of the A- and B-fragment did not prevent reassociation of the constituent fragments of diphtheria toxin. Small amounts of additional bands were also found, possibly representing labelled material that formed disulphide bonds with globin chains in the lysate.

We also made fusion proteins consisting of the B3 tetradecapeptide fused to the *N*-terminus of fragment A (Fig. 8b), of an 82-amino acid piece of dihydrofolate reductase in front of the A-fragment (Fig. 8c), as well as of a 123-amino acid piece of apolipoprotein AI in front of the A-fragment (Fig. 8d) and in front of the B-fragment (Fig. 8j). In the three latter cases the constructs were preceded by the B3 peptide, in order to facilitate identification by immunoprecipitation. When the different proteins were dialysed together with the complementary unmodified fragment, they were found to associate and become linked by a disulphide bond as determined from their lower migration rates in SDS/PAGE. When a fusion protein of whole dihydrofolate reductase and the A-fragment was dialysed together with B-fragment, only small amounts were found migrating at the rate expected for reconstituted toxin (P-DT in Fig. 8e) and most of the material formed did not enter the gel. We have so far not analysed this material further.

It should be noted that reconstituted toxin with ZZ linked to the C-terminus of the A-fragment or to the *N*-terminus of the B-fragment migrated at rates corresponding to approx. 110 kDa and approx. 90 kDa respectively. A possible reason for this is given in the Discussion. In all cases control experiments (not shown) demonstrated that no material migrated at the rates expected for full-length reconstituted proteins when A- and B-fragments or their fusion proteins were dialysed separately. Altogether, the data indicate that formation of diphtheria toxin from the separate fragments is a versatile system to form toxin carrying additional peptide material at different locations in the toxin.

DISCUSSION

The main finding presented here is that separately translated A- and B-fragments of diphtheria toxin react quantitatively to form active diphtheria toxin, provided they are able to form an interfragment disulphide bond. Since very low (nanomolar) concentrations of each fragment were required for efficient association, the A- and B-fragment must have a considerable affinity for each other. On the other hand, in the absence of disulphide formation the association was inefficient, indicating that the interfragment disulphide bridge functions to keep the A- and B-fragment together.

Fig. 8. Reconstitution of toxin from extended A- and B-fragments

[³⁵S]Methionine-labelled reconstituted toxin with different extensions of the A- and B-fragment were analysed by SDS/PAGE under reducing (R) and non-reducing (N) conditions. The gels were submitted to fluorography. The two lanes in each panel represent different lanes from the same gel. The samples in different panels were run on different gels. The locations of fragments and reconstituted toxin are indicated in the following way: P-DT, peptide in front of diphtheria toxin or in internal positions; DT-P, peptide at the C-terminus of diphtheria toxin; P-A, peptide at the N-terminus of the A-fragment; A-P, peptide at the C-terminus of the A-fragment; P-B, peptide at the N-terminus of the B-fragment; B-P, peptide at the C-terminus of the B-fragment.

Reconstitution of diphtheria toxin from its constituent fragments has been carried out previously (Uchida *et al.*, 1972). Two mutant toxins, each of which contained one inactive and one active fragment, were nicked, reduced, mixed and dialysed. Under these conditions active toxin was formed. Isolated fragments have also been reassociated to yield active toxin (Sundan *et al.*, 1982). However, harsh conditions must be used to separate the A- and B-fragments of natural toxin, and the B-fragment

obtained in this way is very unstable. In contrast, B-fragment synthesized in a cell-free system is stable. Possibly, molecular chaperones (Pelham, 1989; Rothman, 1989) present in the lysate stabilize the B-fragment.

As reticulocyte lysate contains large amounts of globin and other SH-containing proteins, one might expect considerable disulphide formation between the separate A- and B-fragments and lysate proteins. Such disulphide formation was, however,

relatively infrequent, indicating that the interaction and disulphide formation between the two fragments is highly specific. When additional peptides were fused to the termini of each fragment, disulphide formation with lysate proteins increased in some cases, suggesting that the specificity of association was reduced.

When protein A-domains were linked to the C-terminus of the A-fragment or to the N-terminus of the B-fragment, the reconstituted toxin migrated more slowly than expected (Fig. 8). This could be due to the fact that the denatured and unreduced protein will have a fork-like structure. They may therefore migrate more slowly in the gel than denatured unreduced normal toxin where the disulphide links the C-terminus of the A-fragment to the N-terminus of the B-fragment such that the denatured molecule may migrate as an almost linear structure.

When dihydrofolate reductase linked to the N-terminus of the A-fragment was dialysed together with B-fragment, the product did not enter the gel. The reason for this could be that the protein is poorly soluble, but so far we have not analysed this in detail. It is likely that not all kinds of peptides and proteins can be added to the A- and B-fragment of the toxin without interfering with reassociation. Only experience will show which conjugates are able to interact with the reciprocal fragment to allow reconstitution to take place. In cases where the reconstitution is inhibited, the use of a flexible linker between the two parts of the fusion protein could relieve the inhibition.

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