Inhibition of Membrane Translocation of Diphtheria Toxin A-fragment by Internal Disulfide Bridges*

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Fragment A of diphtheria toxin is translocated to the cytosol when the toxin is presented to receptor-positive cells. The toxin binds to cell surface receptors through its B-fragment, and after endocytotic uptake, the low endosomal pH triggers translocation of the A-fragment across the membrane. Translocation can also be induced at the level of the plasma membrane by exposure to low pH medium. Based on the diphtheria toxin crystal structure, we made five double cysteine mutants of the A-fragment, each expected to form an intramolecular disulfide bridge. Four of the double cysteine mutants efficiently formed an intramolecular disulfide bridge, and these same mutants showed a strong reduction in their translocating ability. The inhibition of translocation was observed both when the toxin was endocytosed and when translocation was induced by exposing surface-bound toxin to low pH. The data indicate that extensive unfolding of the A-fragment is required for translocation.

How proteins cross biological membranes is a fundamental question in biology, and much research effort has been devoted to resolving the mechanisms of protein translocation. The translocation of proteins from the cytosol to the periplasm of Escherichia coli (for review see Wickner et al. (1991)), and the transport of newly synthesized proteins into mitochondria (reviewed by Glover and Lindsay (1992)) and the endoplasmic reticulum (for review see Nunnari and Walter (1992) and Rapoport (1992)) have been extensively studied. Three characteristic features are shared by these processes. 1) Translocated proteins contain N-terminal targeting sequences that are recognized by receptors at the membrane. 2) A driving force in the form of metabolic energy, and in some cases also a membrane potential, is required. 3) Proteins need to be maintained in a loosely folded state in order to be translocation-competent.

Several protein toxins are able to translocate a catalytic domain to the cytosol of eukaryotic cells. The molecular mechanism of the translocation process has been most thoroughly studied in the case of diphtheria toxin. This toxin is secreted from Corynebacterium diphtheriae as a polypeptide of 58 kDa (Greenfield et al., 1983), which is easily cleaved by low concentrations of trypsin, giving two fragments, A (21 kDa) and B (37 kDa), held together by a disulfide bond (Pappenheimer, 1977). Entry of diphtheria toxin into cells is initiated by binding of the B-fragment to specific cell surface receptors (Uchida et al., 1972), followed by endocytosis, and the low pH in endosomes induces the A-fragment to translocate to the cytosol (Sandvig et al., 1984). Once in the cytosol, the A-fragment ADP-ribosylates elongation factor 2 (Collier, 1975), leading to inhibition of protein synthesis. Translocation can also be induced at the level of the plasma membrane by exposing cells with bound toxin to low pH medium, thereby mimicking conditions inside the endosome (Draper and Simon, 1980; Sandvig and Olanes, 1980).

Translocation of diphtheria toxin A-fragment to the cytosol occurs in the opposite direction compared to the other translocation processes mentioned. Also, several other characteristic features of protein translocation out of the cytosol do not apply to diphtheria toxin. Targeting of the toxin to the plasma membrane is provided for by an entire domain consisting of the ~250 C-terminal amino acids of the B-fragment (Choe et al., 1992; Esbensen et al., 1993), whereas export proteins and mitochondrial proteins typically contain short N-terminal targeting sequences. When translocation is induced by exposure of cells with bound toxin to low pH medium, neither a membrane potential (Sandvig et al., 1986) nor metabolic energy (Moskaug et al., 1988) appears to be required for the translocation process. The diphtheria toxin molecule has acquired its native conformation before its translocation is initiated, whereas proteins that are translocated out of the cytosol are either translocated co-translationally or are (often) kept in a translocation-competent state by molecular chaperones.

Exposure to low pH induces partial unfolding of the toxin molecule (Dumont et al., 1988; Jiang et al., 1991), and it is conceivable that unfolding of the A-fragment is a prerequisite for translocation to occur. The A-fragment does not contain any internal disulfide bonds, and we reasoned that if it must unfold in order to be translocated, the introduction of an artificial disulfide bond could prevent unfolding and inhibit the translocation process. On the basis of the crystal structure of the toxin (Choe et al., 1992), we have identified pairs of amino acids that are positioned sufficiently close in space to be likely to form disulfide bonds when mutated to cysteines. We constructed five different double cysteine mutants of the A-fragment and studied their abilities both to form intramolecular disulfide bonds and to be translocated to the cytosol.

MATERIALS AND METHODS

Buffers and Media—Dialysis buffer consisted of 140 mM NaCl, 20 mM HEPES, and 2 mM CaCl2, adjusted to pH 7.0 with NaOH. Reaction buffer for ADP-ribosylation measurements consisted of 20 mM HEPES,
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pH 7.5, 0.5 mM EDTA, 10 mM DTT, 10 μg/ml bovine serum albumin, 20 μg/ml PMSF, 4 μg/ml soybean trypsin inhibitor, 4 μg/ml aprotinin, 4 μg/ml leupeptin, and 4 μg/ml peptatin A. HEPES medium consisted of bicarbonate- and serum-free Eagle’s minimal essential medium buffered with HEPES to pH 7.4. Lysis buffer consisted of 0.1 M NaCl, 20 mM NaH₂PO₄, 10 mM Tris, 100 μM Na3VO₄, washed three times in HEPES medium, and lysed for 10 min on ice in lysis buffer. The cell lysate was transferred to an Eppendorf tube, nuclei were removed by centrifugation, and cellular protein was precipitated with HEPES medium containing 1 mg/ml PMSF. After centrifugation, the trichloroacetic acid pellet was washed in ether and subjected to SDS-PAGE.

**Construction of Mutant Plasmids—** E. coli strain TG-1 was used as a host for M13mp18 during mutagenesis, and E. coli strain DH5α was used in all cloning procedures. The 60 bp pair of HindIII fragment from the plasmid pKD-52 (Arai et al., 1992), encoding the diphtheria toxin A-fragment, was cloned between the EcoRl and SalI sites in the polylinker region of M13mp18. The indicated mutations were introduced into the A-fragment using a mutagenesis kit from Amersham, United Kingdom, and the following complementary mutant primers: K24C, CATACAGCAAGAGTCCCCGTCG; K24S, CATACAGCAATGATCCCGTG; N85C, GGTGCTGTTATACGCTGTAT; N86C, GGTGCTGTTATACGCTGTAT; K76C, CCA-GAGGCTCTGCACGTCAGAGGCTG; N95C, GGTGCTGTTATACGCTGTAT; G119C, ACTCTTCCTGCAGCTGCTTCC; K126C, AGAGTGTGTTTACATGCTGCTTCC; N142C, GGTGCTGTTATACGCTGTAT; G171C, ACTCTTCCTGCAGCTGCTTCC; N188C, GGTGCTGTTATACGCTGTAT; G194C, ACTCTTCCTGCAGCTGCTTCC; N220C, GGTGCTGTTATACGCTGTAT; G377C, ACTCTTCCTGCAGCTGCTTCC. For expression of the mutant A-fragment the plasmid pBD-23 (Stenmark et al., 1992) was used.

**Cell Cultures—** Vero cells were propagated as earlier described (Sandvig and Olsnes, 1980). For binding and Pronase protection experiments, cells were seeded into 12-well Costar (Cambridge, MA) microtiter plates at a density of 2 × 10⁵ cells/well 1 day prior to the experiments. For toxicity experiments, cells were transferred to 24-well plates at a density of 5 × 10⁴ cells/well 1 day prior to the experiments.

**In Vivo Translation and Characterization of Mutant Proteins—** We constructed five double cysteine mutants of the A-fragment, referred to as CC1 through CC5 (Fig. 1A). The expected disulfide bonds are indicated in a linear representation of the A-fragment (Fig. 1B) and in the diphtheria toxin crystal structure (Fig. 1C). We also constructed five control mutants, where one of the cysteines was replaced by serine. These are referred to hereafter as CS1 through CS5.

Proteins with internal disulfide bonds usually migrate more rapidly in SDS-PAGE than their reduced counterparts. To test if the double cysteine mutants formed intramolecular disulfide bonds, we compared the migration rates on SDS-PAGE of the unreduced and reduced A-fragments carrying a double cysteine mutation. For this purpose, an additional mutation (C186S) was introduced to remove the cysteine originally present in the wild-type A-fragment, thereby avoiding the formation of disulfide bonds between this residue and the introduced cysteines.

**RESULTS**

**Formation and Characterization of Mutant Proteins—** We constructed five double cysteine mutants of the A-fragment, referred to as CC1 through CC5 (Fig. 1A). The expected disulfide bonds are indicated in a linear representation of the A-fragment (Fig. 1B) and in the diphtheria toxin crystal structure (Fig. 1C). We also constructed five control mutants, where one of the cysteines was replaced by serine. These are referred to hereafter as CS1 through CS5.

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As shown in Fig. 2A, in each case the non-reduced A-fragments migrated more rapidly than the reduced counterparts. An exception is CC4, where a substantial part of the A-fragment migrated at the slow rate in the unreduced preparation. This indicates that disulfide formation is less efficient in this mutant than in the other ones. Later refinements of the coordinates for the toxin crystal structure showed that in the case of CC4 the geometry of the two residues mutated was less in favor of forming a disulfide bond than in the other cases.

We also compared the migration rates of the double cysteine mutants with those of their respective control mutants, where one of the two cysteines had been replaced by serine. Under

1 The abbreviations used are: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; NEM, N-ethylmaleimide; MES, 2-(N-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; EF-2, elongation factor 2.
Reducing conditions each of the putative disulfide-forming mutants, CC1–CC5, migrated at the same rate as its respective control mutant (Fig. 2B), whereas under non-reducing conditions (Fig. 2C) the double cysteine mutants in four out of five cases migrated faster than their respective controls, as expected if CC1, CC2, CC3, and CC5 all efficiently formed intramolecular disulfide bonds. In the case of CC4, two bands were observed under non-reducing conditions, similarly to what is shown in Fig. 2A.

The cloning of full-length diphtheria toxin in E. coli is considered hazardous. However, recombinant full-length toxin can be obtained by mixing separately expressed A- and B-fragments together under reducing conditions, followed by removal of the reducing agent by dialysis, allowing disulfide formation between Cys186 in the A-fragment and Cys201 in the B-fragment (Stenmark et al., 1992). Possibly, any of the cysteine residues introduced into the A-fragment by mutagenesis could form a disulfide bond with one of the three cysteine residues present in the B-fragment, and the mutant A-fragments were tested for their ability to associate with the B-fragment. While the C186S-mutated versions of all the 10 mutants listed in Fig. 1A were unable to associate with the B-fragment, all those containing Cys-186 associated efficiently with the B-fragment (data not shown). Clearly, only Cys-186, and none of the cysteine residues introduced by mutagenesis, is able to form a disulfide bridge with the B-fragment.
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The introduced disulfide bridges could distort the conformation of the toxin, e.g. by imposing a constraint on its folding. We therefore studied the resistance to proteinase K of the mutant toxins that had been reconstituted from in vitro translated \(^{35}\)Smethionine-labeled A- and B-fragments. The results (Fig. 3) showed that some of the mutants were slightly more susceptible to proteinase K than wild-type toxin. Importantly, in all cases the disulfide-forming mutants showed basically the same proteinase K sensitivity as their respective serine controls, indicating that it was the introduced mutations, rather than the disulfide bonds as such, that in some cases led to the slight increase in protease susceptibility.

Cell Binding and Membrane Translocation of Mutant Toxins—The B-fragment is responsible for binding the toxin to cell surface receptors, and previous studies have shown that mutations in the A-fragment may interfere with the binding properties of the B-fragment (Mekada and Uchida, 1985). To test if the mutated A-fragments affected toxin binding, whole toxin was reconstituted from in vitro translated \(^{35}\)Smethionine-labeled A- and B-fragments, and added to Vero cells, which were subsequently washed and analyzed by SDS-PAGE. The results in Fig. 4A demonstrate that, when reconstituted with B-fragment, all the 10 mutants were able to bind to cells with approximately the same affinity as wild-type toxin. Also, the binding was efficiently competed out by an excess amount of unlabeled wild-type toxin, showing that the mutants bind to specific diphtheria toxin receptors (data not shown).

When cells with bound radiolabeled diphtheria toxin are exposed to low pH to induce translocation of the A-fragment to the cytosol and are subsequently treated with Pronase, only A-fragment that has been translocated to the cytosol is protected, and can be observed after SDS-PAGE and fluorography (Moskaug et al., 1988). Such experiments were carried out to test the translocation capability of the reconstituted toxins containing an internal disulfide bond in the A-fragment. All the four mutants that efficiently formed internal disulfide bridges (CC1, CC2, CC3, and CC5) were translocated much less efficiently than the respective serine mutants (Fig. 4B). In only one case (CS5) did a control mutant show reduced translocation efficiency compared to the wild-type A-fragment. The mutant CC4, which is inefficient in forming an intramolecular disulfide bond, showed no substantial reduction in its translocating efficiency. The results indicate that an SS bridge within the A-fragment strongly inhibits its translocation to the cytosol. Although not visible in Fig. 4B, a small and variable amount of protected A-fragment was observed in some experiments in the case of the mutants CC1, CC2, and CC5. However, in all cases this amount was much smaller than what was observed with the corresponding control mutants. We believe that this variation may be due to a small and variable fraction of the A-fragments not forming an intramolecular disulfide bond, or a varying ability of the cells to reduce a small part of the bound A-fragments at the cell surface.

Cytotoxicity and ADP-ribosylating Activity of Mutant Toxins—The translocation of endocytosed toxin can be studied indirectly by measuring the ability of the toxin to inhibit cellular protein synthesis. Vero cells were incubated overnight with the mutant toxins, and their ability to incorporate \(^{3}H\)leucine was then measured (Fig. 5A). With the exception of the mutant pair CC4/CS4, all serine mutants were more toxic than the double cysteine mutants. CC4 was ~10-fold more toxic than CS4 (see below). The cytotoxicity was also measured in an assay similar to the translocation experiment in Fig. 4B. Low pH was used to induce the translocation of surface-bound toxin, followed by overnight incubation of the cells in the presence of monensin and anti-diphtheria toxin serum, preventing entry of toxin from endosomes, and finally the ability of the cells to
Fig. 5. Cytotoxicity (A) and ADP-ribosylating activity (B) of mutant toxins. In each panel is shown a disulfide forming mutant (△), its Cys → Ser control mutant (●), and wild type toxin (●). Panels 1, CC1 and CS1; panels 2, CC2 and CS2; panels 3, CC3 and CS3; panels 4, CC4 and CS4; panels 5, CC5 and CS5. A, Vero cells were incubated overnight with increasing concentrations of unlabeled toxin reconstituted from in vitro translated A- and B-fragments. Following the overnight incubation, the cells were incubated for 30 min at 37 °C in medium containing 2 μCi/ml [3H]leucine, and then washed twice with 5% trichloroacetic acid. The cells were dissolved in 100 mM KOH, and the cell-associated radioactivity was measured. B, ADP-nbosylation reactions were run at 25 °C in 50 μl of reaction buffer, containing in vitro translated A-fragment (0.5 nM), EF-2 (0.25 μM), and [adenylate-32P]NAD (800 Ci/mmol, 0.13 μM), and 10-μl samples were removed at different times and precipitated with 10% trichloroacetic acid. The samples were washed with 5% trichloroacetic acid and then with acetone and then subjected to SDS-PAGE. A PhosphorImager screen was exposed to the dried gels for 2 h.

incorporate [3H]leucine was measured. When comparing the double cysteine mutants with their respective control mutants, the results were qualitatively the same as those of Fig. 5A, except that CC4 and CS4 now were equally toxic (data not shown). Possibly, instability of the mutant CS4 in endosomes accounts for the 10-fold difference in toxicity between CC4 and CS4 that was observed when the toxin was endocytosed. Also, the differences in toxicity between the mutants CC1, CC2, and CC3 and their respective serine controls was smaller (5-10-fold difference) when the translocation was induced by low pH than when the toxin was endocytosed.

Only in one case (CS2) was the serine mutant equally toxic as wild-type toxin. The reason for the reduced activity in the other mutants could be impairment of the ADP-ribosylating activity of the A-fragment by the mutations. To test this we measured the ability of the mutants to incorporate [32P]ADP-ribose into EF-2. The results (Fig. 5B) showed that three of the mutant pairs (CC2/CS2, CC4/CS4, and CC5/CS5) displayed approximately the same kinetics for the ADP-ribosylation reaction as the wild-type A-fragment, whereas the two remaining pairs (CC1/CS1 and CC3/CS3) exhibited reduced activity (5-10% of the wild-type A-fragment). Importantly, in this assay there were no detectable differences between the double cysteine mutants and their respective Cys → Ser controls, indicating that the differences in toxicity are due not to differences in enzymatic activity, but to different abilities to translocate across the endosomal membrane.

**DISCUSSION**

Four out of the five double cysteine mutants made were able to efficiently form a disulfide bridge within the A-fragment, and in all these cases the disulfide bond was strongly inhibitory to the translocation process. Two of the translocation-blocking disulfide bonds (CC1 and CC3) are located within one of the two subdomains that constitute the A-fragment, one (CC5) is located within the other subdomain, and one (CC2) connects the two subdomains. The data therefore suggest that the A-fragment must undergo extensive unfolding of its tertiary structure in order to be translocated to the cytosol.

Only in the case of CS5 did a serine control mutant show a reduction in its translocation efficiency. It therefore appears that a number of point mutations can be introduced into the A-fragment without affecting its translocating capability. The reduction in toxicity of those mutants that did not show a reduced translocation efficiency can be satisfactorily explained by the ADP-ribosylation data, except in the case of CS4 and CC4, where the toxicity was reduced, although the measured ADP-ribosylating activity was the same as that of wild-type A-fragment. Conceivably, the stability of these mutants in the endocytic pathway or in the cytosol is reduced.

A number of studies have indicated that tight folding is in general inhibitory for translocation of proteins across membranes. The structure of many proteins can be stabilized by the binding of small ligands, leading to a block in translocation...
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Eilers and Schatz, 1986; Chen and Douglas, 1987; Rassow et al., 1989; Arkowitz et al., 1993). Also, heparin blocks the translocation to the cytosol of a fusion protein between a heparin-binding growth factor and the diphtheria toxin A-fragment (Wiedlocha et al., 1992). Furthermore, internal disulfide bonds have been observed to block translocation of proteins across membranes. Preprolactin is only translocated across microsomal membranes in vitro if internal disulfide bridges are reduced (Maher and Singer, 1986). In a fusion protein between the B subunit of cholera toxin and Neisseria IgA protease, the presence of a disulfide bridge in the toxin B subunit halves translocation of the fusion protein across the outer membrane of E. coli (Klauser et al., 1992). The disulfide bridge between Cys-290 and Cys-302 in the E. coli outer membrane protein OmpA cross-links 2 residues that are located within a relatively short distance along the polypeptide chain, and this disulfide bond is inhibitory for translocation across the inner membrane in the absence, but not in the presence, of a proton motive force (Tani et al., 1990). Bovine pancreatic trypsin inhibitor is a compactly folded protein, containing three disulfide bridges, and when it is linked to a mitochondrial precursor protein, import into the mitochondrial matrix is blocked (Vestweber and Schatz, 1988).

There are many differences between diphtheria toxin translocation into the cytosol and the translocation of newly synthesized proteins out of the cytosol. Therefore it was not obvious that the requirements for unfolding observed in other systems would also apply to diphtheria toxin translocation. A number of other protein toxins are translocated to the cytosol and act enzymatically on intracellular targets, and the enzymatic domains of these protein toxins all share the feature that they do not contain any intramolecular disulfide bonds. Therefore, the observation that unfolding of diphtheria toxin is required for its translocation suggests that this is also the case for translocation of other protein toxins.

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