Introduction of a Disulfide Bond into Ricin A Chain Decreases the Cytotoxicity of the Ricin Holotoxin*

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Wild type ricin A chain (RTA) contains two cysteine residues (Cys171 and Cys269). Cys269 forms the interchain disulfide bond of ricin holotoxin with Cys171 of ricin B chain (RTB). We have used site-directed mutagenesis of RTA cDNA to convert Cys171 to Ser and to introduce a disulfide bond into RTA by converting Ser269 and Met235 to Cys residues. Mutant RTA was expressed in Escherichia coli and directed to the oxidizing environment of the periplasmic space where the Cys269-Cys235 disulfide bond was formed. The disulfide-containing RTA mutant had an in vitro catalytic activity similar to that of an identical form of recombinant RTA that lacked the S215C and M255C mutations. In the presence of glutathione and protein disulfide isomerase, in this RTA variant reassociated with RTB to form ricin holotoxin. Incubation of this holotoxin with increasing concentrations of dithiothreitol showed that the interchain disulfide bond joining RTA and RTB was more readily reduced than the intrachain disulfide bond in RTA. Ricin in which the RTA moiety contained the disulfide bond was 15-18-fold less cytotoxic to HeLa or Vero cells than ricin in which the RTA did not contain the stabilizing disulfide cross-link. Since these ricin molecules had identical RTB cell binding and RTA catalytic activities, we suggest that the observed reduction in cytotoxicity caused by the introduced disulfide bond resulted from a constraint on the unfolding of RTA, indicating that such unfolding is necessary for the membrane translocation of RTA during its entry into the cytosol.

In ricin, the toxic polypeptide (the A chain, or RTA)1 is joined to a galactose-binding polypeptide (the B chain, or RTB) by a disulfide bond. RTB is responsible for binding ricin to the cell surface and is also believed to facilitate endocytic transport to the cellular compartment most conducive for the subsequent membrane translocation step (6). Since there is no evidence for the translocation of RTB along with RTA (indeed, RTB can be successively replaced by other cell binding proteins such as antibodies (2)), it is generally assumed that ricin becomes reductively cleaved within the endomembrane system prior to membrane traversal. Neither the site nor the mechanism of membrane translocation have yet been identified. We assume, however, that the translocation step requires that RTA becomes at least partially unfolded. If this is the case then any constraint on unfolding might be expected to reduce the apparent cytotoxicity of ricin if the amount of RTA in the translocation-ally competent compartment is limiting. To test this we have engineered an intrachain disulfide bond into RTA. In the present paper we show that ricin containing mutant recombinant RTA with an introduced disulfide bond is less cytotoxic than ricin containing an identical recombinant RTA but lacking this stabilizing disulfide cross-link.

MATERIALS AND METHODS

Creation of Mutants—The expression vector pKK223.3 was digested with EcoRI and HindIII, and the large (4555-base pair) fragment was isolated. An irrelevant DNA fragment was inserted to maintain the restriction sites, the plasmid was digested with BamHI and SalI, and 5' overhangs were removed and religated to produce plasmid pKH50, which no longer possessed tetracycline resistance or BamHI and SalI sites. Two complementary synthetic oligonucleotides with 5' overhangs, encoding the Escherichia coli ompF signal sequence and EcoRI, XhoI, BamHI, and HindIII restriction sites, were annealed, end-filled, and cloned into pKH50 to form pKH100. An RTA cDNA clone, containing an XhoI site at the 5'-position of the preproricin N-terminal extension and a stop codon directly after the RTA coding sequence (7) was cloned into pKH100 via the XhoI and BamHI sites to create pFAC.

Mutagenesis of the RTA cDNA was performed by standard techniques using an oligonucleotide-directed in vitro mutagenesis kit (U. S. Biochemical Corp.) as instructed by the manufacturer. Mutations were verified by dideoxy sequencing. Mutagenic oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer and were designed such that the mismatch(es) to be introduced was flanked on either side by at least seven bases complementary to the wild type sequence.

Expression and Purification of Recombinant Proteins—E. coli TG2 cells (8) transformed with the appropriate vector were inoculated in defined M9 medium to give an A600 of 0.1. The culture was grown with shaking at 37 °C until the A600 reached 0.7-0.8. Expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mm, and the culture was shaken for 3 h at 30 °C. After harvest,* this work was supported by Grant GR/G00877 from the United Kingdom Science and Engineering Research Council Biotechnology Directorate. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: RTA, ricin A chain; RTB, ricin B chain; DTA and DTB, diphtheria toxin A and B fragment respectively; TBS, Tris-buffered saline; MAP, Mirabilis antiviral protein; rRTA, recombinant RTA.
vesting, cells were resuspended in 580 mM sucrose, 300 mM Tris, pH 8.0, 1 mM EDTA, 0.5 mM MgCl₂, allowed to stand on ice for 10 min, and then repelleted by centrifuging at 7500 rpm (6200 g) for 5 min. Cells were resuspended in 1 mM Tris, pH 7.5, and after a further 10 min on ice were centrifuged at 19,000 rpm (28,000 x g) and 4 °C for 15 min. The supernatant, which contained the periplasmic fraction, was dialyzed three times against 5 mM sodium phosphate, pH 6.5. Recombinant protein from a 1-liter culture was purified by ion-exchange chromatography on a 1.6 x 40-cm CM-Sepharose column in 5 mM sodium phosphate, pH 6.5, and bound proteins were eluted with a 0–300 mM NaCl gradient. Fractions containing recombinant product, determined by SDS-polyacrylamide gel electrophoresis followed by silver staining or Western blotting. Free sulfhydryl groups were blocked by adding 20 ml of 10 mM N-ethylmaleimide in 7.5, 150 mM NaCl) containing 0.1% Tween 20 and once in TBS without sodium phosphate, pH 6.5. Recombinant FACO-RTA, or FAC4-RTA were incubated at room temperature with a 2-fold excess of purified RTB (Inland Laboratories, Austin, TX). Where appropriate, the reassociation mixtures were supplemented with 10 mM glutathione (in a reduced-to-oxidized ratio of 2:1, mimicking that found in the endoplasmic reticulum lumen (10)), 10 mM glutathione plus 1.4 μM protein disulfide isomerase, or ⅓ volume of reticuloplasm. Reticuloplasm, the soluble components of the endoplasmic reticulum lumen, was purified from MOPC-315 cells as described previously (11). In order to separate holotoxin from free RTA or RTA dimers/aggregates, reassociation mixtures were passed down a small column containing 2 ml of SeLectin-2 beads (lactose immobilized on acrylamide) (Pierce). The column was washed with PBS before bound holotoxin was eluted with PBS containing 50 mM galactose. Purified holotoxin was quantified by densitometry of silver-stained SDS-polyacrylamide gels, on which its RTA component was compared to standards of known amounts of purified rRTA, using a Molecular Dynamics computing densitometer.

**Cytotoxicity Assays**—Vero cells or HeLa cells grown in Dubecco’s modified Eagle’s medium containing 5% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin were plated out in 96-well microtiter plates at a density of ~0.5 x 10⁴ cells/well. After allowing time for cells to adhere (at least 4 h) and washing with PBS, various toxin concentrations in 50 μl of media were added to each well, and the cells were incubated at 37 °C for various times or overnight. The cells were then washed with PBS, and protein synthesis was measured by adding 1 μCi of [³⁵S]methionine in 50 μl of PBS to each well and incubating at 37 °C for 2 h. Labeled proteins were precipitated by three washes with 5% (v/v) trichloroacetic acid and after three further washes in PBS were released with 0.2 N NaOH, and the incorporated radioactivity was determined by scintillation counting.

**Other Methods**—DNA manipulations, SDS-polyacrylamide gel electrophoresis, and Western blotting were carried out using standard published procedures (12, 13).

**RESULTS**

The RTA construct used for mutagenesis and subsequent expression is shown in Fig. 1. The RTA coding sequence was altered by the E. coli omplF signal sequence. In constructing the fusion (utilizing the XhoI site at -15-position in preproricin (7)), the RTA sequence codes for 5 additional amino acid residues at the N terminus of RTA that are derived from the preproricin leader sequence. A stop codon was introduced immediately after the RTA coding sequence. Expression was directed by the isopropyl-1-thio-β-D-galactopyranoside-inducible Tac promoter. The plasmid was designated pFAC for omplF-RTA-cysteine mutant.

Wild type RTA contains 2 cysteine residues at positions 171 and 259. To avoid possible thiol/disulfide interchange reactions with introduced disulfides, cysteine 171 was converted to serine by standard site-directed mutagenesis of the encoding DNA (C171S mutation to produce the construct pFAC0). Cysteine 259 was not changed since this residue is required for formation of the interchain disulfide bond linking RTA and RTB in the ricin holotoxin. pFAC0 encodes a variant form of RTA that differs from native RTA in containing five extra N-terminal amino acids and the C171S substitution. RTA encoded by
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pFAC0 (FAC0-RTA) therefore provided the control toxin for comparison with mutant derivatives carrying additional pairs of cysteine residues, since the pFAC0 construct was used as the template for the introduction of these additional cysteine codons. The pairs of FAC0-RTA residues that were converted to cysteine residues, since the pFAC0 construct was used as the template for the introduction of these additional cysteine codons. The pairs of FAC0-RTA residues that were converted to cysteine residues, since the pFAC0 construct was used as the template for the introduction of these additional cysteine codons. The pairs of FAC0-RTA residues that were converted to cysteine residues, since the pFAC0 construct was used as the template for the introduction of these additional cysteine codons.

**Table I**

| Plasmid | Mutant protein | Residue changes | Codon changes |
|---------|----------------|-----------------|---------------|
| pFAC0*  | FAC0-RTA       |                 |               |
| pFAC1   | FAC1-RTA       |                 |               |
| pFAC2   | FAC2-RTA       |                 |               |
| pFAC3   | FAC3-RTA       |                 |               |
| pFAC4   | FAC4-RTA       |                 |               |

*The template for mutagenesis to generate pFAC1–4.

RTA bands were visualized on Western blots of periplasmic preparations by adding sheep anti-RTA antibodies followed by color development (data not shown). The Western blots showed that pFAC, pFAC0, and pFAC4 expressed well. Recombinant products were directed to the periplasmic space by the E. coli ompF signal sequence to facilitate disulfide bond formation (14).

**Fig. 1.** SDS-polyacrylamide gel electrophoresis of purified RTA and RTA mutants. Lanes 1, 2, and 3 were run in the presence of 25 mM dithiothreitol; lanes 4 and 5 were run in the absence of dithiothreitol. Lane 1, wild type RTA; lanes 2 and 4, FAC0-RTA; lanes 3 and 5, FAC4-RTA. Protein bands were visualized by silver staining.

**Fig. 2.** SDS-polyacrylamide gel electrophoresis of purified RTA and RTA mutants. Lanes 1, 2, and 3 were run in the presence of 25 mM dithiothreitol; lanes 4 and 5 were run in the absence of dithiothreitol. Lane 1, wild type RTA; lanes 2 and 4, FAC0-RTA; lanes 3 and 5, FAC4-RTA. Protein bands were visualized by silver staining.

**Fig. 3.** Visualization of intrachain disulfide bonds in proteins using digoxigenin reagent. Proteins were electrophoresed on a non-reducing SDS-polyacrylamide gel, blotted, and treated as described under "Materials and Methods." Lane 1, subtilisin; lane 2, fetuin; lane 3, RTB; lane 4, α-sarcin; lane 5, rRTA; lane 6, FAC4-RTA.

Reassociation experiments with purified FAC4-RTA and biochemically purified RTB produced very little holotoxin when the two proteins were mixed without further additions. This contrasts with a high level of reassociation under the same conditions when native RTA and RTB were mixed (data not shown). The RTA cysteine that forms the disulfide bond with RTB (Cys<sup>259</sup>) is close to the C terminus of RTA and, in FAC4-RTA, is also close to the introduced Cys<sup>259</sup>-Cys<sup>255</sup> disulfide bond. The RTA cysteine that forms the disulfide bond with RTB (Cys<sup>259</sup>) is close to the C terminus of RTA and, in FAC4-RTA, is also close to the introduced Cys<sup>259</sup>-Cys<sup>255</sup> disulfide bond. The RTA cysteine that forms the disulfide bond with RTB (Cys<sup>259</sup>) is close to the C terminus of RTA and, in FAC4-RTA, is also close to the introduced Cys<sup>259</sup>-Cys<sup>255</sup> disulfide bond.
FIG. 4. Depurination of reticulocyte rRNA by RTA and RTA mutants. Ribosomes were incubated for 30 min with equal amounts of periplasm containing toxin variants. RNA was extracted, treated with aniline (+I or not treated), and separated by 1.2% denaturing agarose gel electrophoresis. The arrow indicates the 390-ribonucleotide fragment released by aniline treatment of depurinated RNA. Lanes 1 and 2, FAC-RTA; lanes 3 and 4, FAC0-RTA; lanes 5 and 6, control periplasm from E. coli transformed with plasmid pKK223.3; lanes 7 and 8, FAC4-RTA; lanes 9 and 10, rRTA.

FIG. 5. Inhibition of in vitro protein synthesis in rabbit reticulocyte lysates by wild type rRTA (■), FAC0-RTA ( ), and FAC4-RTA (○).

successful, indicating that the presence of the engineered disulfide bond was not itself limiting reassociation. The addition of 10 mM glutathione (in a reduced-to-oxidized ratio of 2:1 (10)), glutathione plus 1.4 μM protein disulfide isomerase, or 1/4 volume of isolated reticuloplasm (the soluble components of the endoplasmic reticulum lumen from MOPC-315 cells (11)) increased the degree of reassociation significantly. Reassociation in the presence of both glutathione and protein disulfide isomerase produced the highest level of holotoxin (Fig. 6). Holotoxin containing FAC4-RTA was isolated by affinity chromatography on immobilized lactose and eluted with galactose (Fig. 6). This ensured that the holotoxin preparation did not contain FAC4-RTA dimers or aggregates. Likewise, holotoxins containing either wild type rRTA or FAC0-RTA were obtained and affinity purified.

In holotoxin containing FAC4-RTA, the interchain disulfide bond was more easily reduced by dithiothreitol than the intrachain disulfide in FAC4-RTA. At low dithiothreitol concentrations, the small amount of FAC4-RTA reductively released from the holotoxin ran on the gel with a mobility characteristic of the disulfide cross-linked polypeptide (Fig. 7). As the dithiothreitol concentration was increased, more FAC4-RTA was released from the holotoxin, and an increasing proportion of the released protein ran with the lower mobility characteristic of the fully reduced polypeptide (Fig. 7). In the presence of 25 μM dithiothreitol the holotoxin dissociated completely and all of the released FAC4-RTA was reduced (data not shown). When holotoxin containing FAC0-RTA was likewise treated with a range of dithiothreitol concentrations, the released FAC0-RTA ran as a single band whose mobility was identical to that of reduced FAC4-RTA (data not shown).

The cytotoxicities of the holotoxins towards Vero cells were then compared (Fig. 8). Holotoxin containing wild type rRTA was more cytotoxic (with an IC_{50} value of 0.12 ng/ml (Table II)) than holotoxin containing FAC0-RTA (IC_{50} 1.4 ng/ml). Significantly, holotoxin containing FAC0-RTA was 18-fold more cytotoxic than holotoxin containing FAC4-RTA (IC_{50} for FAC4-RTA holotoxin, 25 ng/ml). Similar results were obtained in cytotoxicity assays using HeLa cells (Table II). For the cytotoxicity assay (Fig. 8), each point is the mean value from triplicate samples, and the cytotoxicity profiles obtained were observed in three separate experiments.

DISCUSSION

In the present paper we describe the replacement of pairs of RTA residues with cysteine residues in an attempt to engineer novel disulfide bonds into the protein. The criteria for choosing these cysteine pairs were based on the coordinates of cysteine residues in naturally occurring disulfide bonds from known protein x-ray structures. These criteria were as follows: (i) the chosen cysteine pairs were separated by more than 20 residues of the polypeptide chain; (ii) the Cα-Cα distances fell within the range for naturally occurring disulfide bonds (4.6–6.8 Å) (16); (iii) the target Cys were reasonably juxtaposed so as to allow disulfide bond formation without serious disruption of the backbone; (iv) there was no obvious potential steric hindrance to disulfide bond formation; and (v) neither of the target residues selected for mutagenesis was strictly conserved in the sequence alignment of plant toxin A chains or single chain ribosome-inactivating proteins (17). Such conservation could imply an essential structural or functional role for the residue. Similarly potential disulfide bonds were rejected if one of the target residues was located near the RTA active site cleft.
Fig. 7. Reduction of interchain and intrachain disulfide bonds. Holotoxin containing FAC4-RTA was incubated for 30 min at 37 °C with 0 (lane 1), 0.05 (lane 2), 0.1 (lane 3), 0.5 (lane 4), 1 (lane 5) and 2.5 (lane 6) mM dithiothreitol. 10 mM iodoacetamide was then added and, after a further 10 min at 37 °C, the samples were run on a nonreducing SDS-polyacrylamide gel electrophoresis and Western blotted, and RTA was visualized using sheep anti-recombinant RTA antibodies. These antibodies did not cross-react with RTB.

TABLE II
Cytotoxicity of ricin holotoxin containing wild type or mutant RTA

|                 | Vero cells | HeLa cells |
|-----------------|------------|------------|
| Wild type RTA   | 0.12       | 0.39       |
| FAC0-RTA        | 1.40       | 4.70       |
| FAC4-RTA        | 25.00      | 70.00      |

A further consideration was based on the presumed mechanism of ricin entry into cells. Before translocation into the cytosol occurs, RTA may dissociate from RTB following reduction of the interchain disulfide bond. This implies that the solvent at the site of RTA translocation may carry a reducing potential. The residue pairs listed in Table I were chosen because they should generate disulfide bonds in the polypeptide that are inaccessible to solvent. In this way an engineered disulfide bond ought to represent a stable constraint on protein unfolding.

Of the four cysteine pairs that were introduced into RTA (Table I), only one pair (S215C,M255C) led to the production of significant amounts of soluble, stable, and biologically active mutant product (designated FAC4-RTA) in E. coli. Fig. 9 shows the RTA backbone and the position of the engineered disulfide bond of FAC4-RTA. Both of the introduced cysteines are in reasonably close positions to the naturally occurring Cys255. From the x-ray structure of RTA (18) we concluded that neither Cys215 nor Cys255 were likely to form a disulfide bond with Cys255 (19), leaving it free to interact with Cys4 of RTB during holotoxin formation. Both Cys215 and Cys255 are buried in the RTA molecule, and if a disulfide bond had formed between either of these residues and Cys255, the remaining free cysteine residue would also be buried and would be unable to interact with Cys4 of RTB. On the other hand, FAC4-RTA requires the addition of protein disulfide isomerase and glutathione before it associates to a significant extent with RTB. One possible explanation for this is that the disulfide bond in FAC4-RTA forms between one of the introduced cysteine residues and Cys255 and that this form cannot associate with RTB. Thiol exchange, catalyzed by protein disulfide isomerase, might rearrange the disulfide bond to the Cys215-Cys255 form, which is then capable of associating with RTB via Cys215. However, since FAC0-RTA, which only contains a single cysteine residue (Cys255), also requires protein disulfide isomerase and glutathione to associate effectively with RTB, we feel it is unlikely that the disulfide bond in FAC4-RTA involves Cys255. We are confident, therefore, that the disulfide bond introduced into FAC4-RTA joins Cys215 and Cys255. The introduced disulfide bond should be relatively inaccessible until the protein becomes at least partially unfolded. The current model for ricin cytotoxicity assumes that the reductive release of RTA from the holotoxin precedes the RTA membrane translocation step; RTB is not thought to cross the vesicle membrane. If the internal disulfide bond in FAC4-RTA subsequently constrains its unfolding, then this disulfide bond should be less sensitive to reduction than the disulfide bond joining RTA and RTB. Consistent with this, we observed that the interchain disulfide bond in holotoxin containing FAC4-RTA was more readily reduced than the intrachain disulfide bond present in FAC4-RTA itself (Fig. 7). In keeping with the conclusions of earlier studies, we assume that the folded conformation of RTA would be stabilized by the introduction of the disulfide bond (20, 21). This effect would presumably be largely the result of a decrease in the configurational chain entropy of the unfolded molecule (22, 23).

The introduction of the disulfide bond did not appear to perturb the RTA active site in any way, since FAC4-RTA had catalytic activity similar to that of FAC0-RTA, which lacked the disulfide cross-link (Fig. 5). The translational system used to measure in vitro protein synthesis inhibition contained dithiothreitol, and it is possible that this reducing agent could have broken the disulfide cross-link in FAC4-RTA (Fig. 5). The presence of an intact disulfide bond may well have reduced the catalytic activity of FAC4-RTA in comparison with that of

![Fig. 9. View of the carbon-α backbone of RTA showing the disulfide bond introduced into FAC4-RTA.](image)
FACO-RTA. *M. mirabilis* antiviral protein (MAP) is an RTA-like rRNA N-glycosidase that contains a naturally occurring disulfide bond (24). When a mutant MAP gene, in which the codons for both cysteines of this disulfide bond were replaced by serine codons, was expressed in *E. coli*, the catalytic activity of the mutant protein was 22 times higher than that of native MAP (25). Nevertheless we feel that the activity comparison of FAC4-RTA and FACO-RTA in a reducing translational system (Fig. 5) is justified, since the intracellular site of action of these toxins is the reducing cytosol, where we assume they would also display identical catalytic activity.

The RTA variants were associated with RTB to form holotoxin. The various holotoxins were identical in terms of their RTB-mediated cell binding function and very similar in terms of their RTA catalytic activities (Fig. 5). They were, however, significantly different in terms of their cytotoxicity to Vero cells or HeLa cells (Fig. 8, Table II). The data in Fig. 8 and Table II were based on cytotoxicity assays in which the cells were incubated overnight with toxin. Identical results were obtained when the duration of exposure to toxin was reduced to 3 or 6 h (data not shown). Holotoxin containing wild type RTA was the most potently toxic. In addition to slight differences in catalytic activities (Fig. 5), FACO-RTA and FAC4-RTA both differ from wild type RTA in having 5 extra amino acid residues at the N terminus and the C171S mutation, and this may have reduced their membrane translocation activity as compared with that of wild type RTA. The only difference between the FACO-RTA and FAC4-RTA holotoxins, however, was the disulfide bond in the latter.

Before the cytotoxic proteins enter the cytosol they must cross the membrane of an intracellular compartment. At least partial unfolding of the polypeptide chain is likely to be a prerequisite before membrane translocation can occur. The introduced disulfide bond in FAC4-RTA was expected to constrain the unfolding of FAC4-RTA in comparison with that of FACO-RTA. If this is the case, ricin holotoxin containing FACO-RTA should be more cytotoxic than an equivalent concentration of ricin containing FAC4-RTA when the toxin concentration in the translocation compartment is limiting. Fig. 8 shows that this predicted outcome was observed experimentally. The introduced disulfide bond of FAC4-RTA would not be expected to inhibit either cell binding or intracellular uptake and transport of whole ricin, events that are mediated by RTB. Further, it does not appear to inhibit the catalytic activity of RTA, at least in a reducing environment similar to that of the cytosol (Fig. 5). The most likely explanation for the reduction in ricin cytotoxicity resulting from the introduction of a disulfide bond into RTA is that it impedes the membrane translocation step. Whereas alternative explanations cannot be discounted at present, this impedance probably results from an additional constraint on the unfolding of RTA prior to or during translocation. If this proves to be the case, then the present results also imply that the unfolding of RTA is a necessary prerequisite for entry into the cytosol.

While the present paper was in preparation, a separate study reported that the introduction of internal disulfide bonds into the A fragment of diphtheria toxin also significantly decreased the cytotoxicity of the holotoxin (26). DTA translocation occurs from acidified endosomes by a mechanism that depends on the low pH environment (27). Acidification induces a conformational change in DTA, allowing it to insert into the endosomal membrane to create a translocation pore for DTA (28). Translocation of DTA across the plasma membrane of cells can be induced by exposing cells with surface-bound toxin to low pH medium, which reproduces the conditions normally encountered by the toxin within endosomes (29, 30). This direct translocation assay was employed to show that the introduced disulfide bonds inhibited membrane translocation by constraining the unfolding of DTA (26). A direct translocation assay is not available in the case of ricin. RTA translocation differs from that of DTA in that it occurs from a different intracellular compartment (31), it does not require a low pH environment (32), and RTB is unlikely to be a component of a membrane translocation channel. Despite differences in the site and mechanism of membrane translocation for RTA and DTA, it appears that both toxin polypeptides must unfold to traverse the membrane. Such unfolding is probably necessary for all protein toxins that reach the cytotoxic site of target cells.

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