Immunochemical Analysis of the Structure of Diphtheria Toxin Shows All Three Domains Undergo Structural Changes at Low pH*

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Diphtheria toxin is a bacterial protein that undergoes a physiologically critical conformational change at low pH. This change involves a partial unfolding event forming a molten globule-like structure, which exposes hydrophobic regions and which allows the toxin to insert into, and translocate across, membranes. In this report, antibody binding was used to examine the regions of the toxin that undergo structural changes at low pH. Monoclonal antibodies specific to the catalytic (C), transmembrane (T), and receptor-binding (R) domains of diphtheria toxin were prepared and isolated. In addition, the binding of anti-peptide antibodies raised against peptides in the C and T domains to toxin was examined. Anti-C monoclonals and antipeptide antibodies were found to bind preferentially to low pH-treated toxin relative to native toxin. Anti-T and anti-R monoclonal binding ranged between preference for native toxin and preference for low pH-treated toxin. These results suggest that the C domain becomes more exposed to solution at low pH, and that both the T and R domains of the B chain undergo major conformational changes at low pH. Based on these results, a model in which low pH induces several coordinated changes in intra- and interdomain interactions is suggested. The participation of the R domain in these changes is of particular significance because it suggests that the R domain plays a more important role in low pH-induced changes than previously realized.

EXPERIMENTAL PROCEDURES

Materials—ImmunoPlate MaxiSorp, flat-bottom 96-well microtiter plates were purchased from Nunc. Goat anti-mouse Ig conjugated to alkaline phosphatase was purchased from Fisher Biotech. Goat anti-rabbit Ig conjugated to alkaline phosphatase was purchased from Southern Biochemical Associates (Birmingham, AL). Horse anti-diphtheria toxin Ig was purchased from Connaught Laboratories (Swiftwater, PA). Rabbit anti-horse Ig conjugated to alkaline phosphatase was purchased from Sigma. Gelatin was purchased from Difco. Sigma 104 phosphatase substrate (5-mg tablets), BCIP, and NBT were purchased from Sigma. PhastSystem SDS-polyacrylamide gel electrophoresis gels and supplies were obtained from Pharmacia Biotech Inc. Nitrocellulose membranes were purchased from Schleicher & Schuell. Low molecular weight standards were purchased from Electran (Poole, United Kingdom).

Immunization of Mice—Formalized diphtheria toxin was prepared 5,5′-diphenyl-3,3′-[3,3′-dimethoxy-4,4′-diphenylene)ditetrazoliumchloride or nitro blue tetrazolium, PBS, phosphate-buffered saline, BSA, bovine serum albumin.

1 The abbreviations used are: C, catalytic; T, transmembrane; R, receptor-binding; ApUp, adenosine-3′-phosphate 5′-uridine phosphate; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; NBT, 2,2′-di-p-nitrophenyl-

5,5′-diphenyl-3,3′-[3,3′-dimethoxy-4,4′-diphenylene)ditetrazoliumchloride or nitro blue tetrazolium, PBS, phosphate-buffered saline, BSA, bovine serum albumin.

2 We have changed the names we established previously (7) for the conformations of toxin exposed to low pH and then pH neutralized from R′ and R to L′ and L″ (where L stands for low pH-treated, and r for reversed to neutral pH), respectively. This was done to avoid confusion between the R domain and R conformation.
by incubating native toxin with 0.2% (v/v) formalin for 1 week at room temperature (8) or unformalized free dimer toxin was prepared by incubating toxin at low pH and 41 °C for 30 min. The toxin sample was mixed with the adjuvant RAS (Ribi Immunocchemicals Research, Hamilton, MT), and injected into BALB/c mice every 2 weeks for 2 months. The hybridomas were produced from these mice by the Tissue Culture Laboratory, Microbiology Department, SUNY, Stony Brook by standard procedures (9).

Screening Hybridomas—Supernatants from hybridomas were screened by ELISA and Western blotting. The ELISA protocol used was similar to that described above, except that the microtiter plate was incubated at 37 °C and supernatants were not preincubated with toxin in the Lr conformation. After preliminary experiments, six hybridoma supernatants we named anti-(α–C1), anti-(α–C2), anti-(α–T1), anti-(α–T2), anti-(α–R1), and anti-(α–R2) were chosen for further study and then subcloned by limited dilution in HyClone fetal bovine serum. All except α-C1 antibody were derived from mice injected with the formalinized toxin.

Antibody Isotyping—Antibody class and subclass were determined using a mouse-hybridoma subtyping kit purchased from Bio-Rad laboratories. The only contaminant appeared to be a light chain. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21). The protein concentration of the stock solutions was 0.02% NaN₃, pH 7.0. The protein concentration of the stock solutions was determined by the Lowry method (21).
RESULTS

Epitope Mapping—Western blotting was used to epitope map monoclonal antibody binding sites on toxin molecules using reduced toxin (which gives bands for the A and B chain as well as whole unnicked toxin), isolated T domain, and isolated R domain (Fig. 1). Three types of antibodies were found: anti-A chain antibodies, which bound to whole toxin and A chain (C domain); anti-T domain antibodies, which bound whole toxin, B chain, and isolated T domain; and anti-R domain antibodies, which bound to whole toxin, B chain and isolated R domain. Two antibodies against each domain (named α-C1, α-C2, α-T1, α-T2, α-R1, α-R2, anti-toxin polyclonal).

The amount of antibody bound to the wells is assayed by the

extracellular binding of the previously prepared (10) anti-peptide antibodies, which bound to the 1–161 fragment of the A chain, but mainly bind to residues 141–157 of the toxin sequence, and thus antibody concentration was 0.48 μg/ml.

α-R2 binding to isolated R domain in the native and Lr' conformation was compared. The Lr' conformation was prepared by adding 5 μl of 1.4 mg/ml T domain to 87.5 μl of 20 mM sodium acetate, 300 mM NaCl, pH 4.4, for 30 min at room temperature, and then neutralized with 214 μl of PBS. The same acetate and PBS were premixed and then added to the toxin to obtain native toxin. Aliquots were diluted to 120 μl with the same mix of buffers and then to 320 μl with PBS and antibody as above. Final antibody concentration was 4.7 μg/ml.

α-T224–237 binding to isolated T domain in the native and Lr' conformation was compared. The Lr' conformation was prepared by adding 27.5 μl of 0.2 mg/ml R domain to 110 μl of 20 mM sodium acetate, 300 mM NaCl, pH 4.4, for 30 min at room temperature and then neutralized with 257 μl of PBS. The same acetate and PBS were premixed and then added to the T domain to obtain native T domain. Aliquots were diluted to 225 μl with the same mix of buffers and then to 320 μl with PBS and antibody as above. Final antibody concentration was 0.48 μg/ml.

Epitope mapping of monoclonal antibodies. Antibody binding to individual domains was assayed by Western blotting of SDS-polyacrylamide gels. Lane 1 contains samples of reduced whole toxin, and has bands corresponding to unnicked whole toxin, B chain (R + T domains), and A chain (C domain). Lane 2 contains samples of isolated T domain. Lane 3 contains samples of isolated R domain. The polyclonal antibody blot shows reaction with all of the molecules; whole unnicked toxin, B chain, and A chain in lane 1; T domain monomers and dimers in lane 2; and R domain monomers and dimers in lane 3. Antibodies used were: α-C1, α-C2, α-T1, α-T2, α-R1, α-R2, anti-toxin polyclonal domain was prepared by mixing 2 μl of 1.29 mg/ml C domain and 249 μl of PBS. The Lr' conformation was toxic prepared by mixing 3.5 μl of Apu-p-bound monomer with 35 μl of 20 μM sodium acetate, 300 mM NaCl, pH 4.4, for 30 min at room temperature, and then neutralizing with 214 μl of PBS. The same acetate and PBS were premixed and then added to the toxin to obtain native toxin. Aliquots were diluted to 120 μl with the same mix of buffers and then to 320 μl with PBS and antibody as above. Final antibody concentration was 0.48 μg/ml.

α-T224–237 binding to isolated T domain in the native and Lr' conformation was compared. The Lr' conformation was prepared by adding 5 μl of 1.4 mg/ml T domain to 87.5 μl of 20 mM sodium acetate, 300 mM NaCl, pH 4.4, for 30 min at room temperature and then neutralized with 257 μl of PBS. The same acetate and PBS were premixed and then added to the T domain to obtain native T domain. Aliquots were diluted to 225 μl with the same mix of buffers and then to 320 μl with PBS and antibody as above. Final antibody concentration was 4.7 μg/ml.

α-R2 binding to isolated R domain in the native and Lr' conformation was compared. The Lr' conformation was prepared by adding 27.5 μl of 0.2 mg/ml R domain to 110 μl of 20 mM sodium acetate, 300 mM NaCl, pH 4.4, for 30 min at room temperature and then neutralized with 257 μl of PBS. The same acetate and PBS were premixed and then added to the R domain to obtain native R domain. Aliquots of both preparations were then made up to 220 μl with the same mix of buffers and then diluted to 220 μl with PBS and antibody as above. Final antibody concentration was 0.48 μg/ml.

Higher resolution epitope mapping was performed with trypsin-digested toxin. Incubating native toxin with trypsin results in the cleavage of the C domain between Lys-39 and Ser-40 (12). Western blotting of trypsin-digested toxin showed monoclonal anti-C domain antibodies did not bind to the 40–193

3 α-C141–157 was generated against a peptide corresponding to residues 141–157 of the toxin sequence, but mainly binds to residues 145–149. α-T224–237 was generated against a peptide corresponding to residues 224–237 of the toxin sequence, but mainly binds to residues 231–236 (10).

fragment (Fig. 2), although as expected α-C141–157 did bind. This suggests part of the epitope for α-C1 and α-C2 lies on residues 1–39, although not all of the epitope may be within this region.

Epitopes were also mapped with cysteine cleavage reagent 2-nitro-5-thiocyanobenzoic acid. Antibody binding to cleavage products of mutant toxins in which a single cysteine was substituted for either Glu-162 or Ser-337 was examined. Anti-C antibodies bound to the 1–161 fragment, but not to the 162–535 fragment, and anti-T domain antibodies bound to the 1–336 fragment but not the 337–565 fragment (data not shown). This suggests that the anti-C domain antibodies bind between residues 1 and 161, and the anti-T domain antibodies bind between residues 194 and 336.

Additional data on epitope locations comes from the results of antibody binding to octapeptides that cover the entire sequence of the B chain (10). The α-R1 antibody binding profile shows an affinity for octapeptides with sequences identical to the loop region between the T and R domain and residues 486–500 (RB8) (data not shown). The binding profile of α-R2, together with hydroxylamine cleavage patterns (data not shown), suggests α-R2 binds residues 454–465 (RB6).

The Effect of pH-induced Changes in Toxin Conformation on Antibody Binding: Competition ELISA—Diphtheria toxin undergoes a conformational change at pH 5.3 that plays a critical role in its entry into cells (4) (see Introduction). Above pH 5 the toxin remains in the native (N) state, but after exposure to low pH at 23 °C, the toxin undergoes a partial unfolding process that results in its taking on a hydrophobic, membrane-inserting conformation (5–7, 13–20). In this conformation, the B chain has undergone a partial unfolding process but the C domain remains folded (7). In order to further characterize the changes that the toxin undergoes at low pH, the pH dependence of antibody binding to toxin was studied. As with many antibodies, anti-toxin binding at low pH was found to be too weak to measure. Therefore, binding was examined after low pH was reversed to neutral. Under these conditions previous studies have shown the toxin maintains the structural changes that occur at low pH (7). This low pH-treated state is called the Lr' conformation.3

To measure the dependence of antibody binding on the pH at which toxin is incubated, toxin was preincubated at various pH values in solution and its antibody binding measured by a competition ELISA after pH neutralization. In this assay the amount of antibody binding to the toxin in solution is measured by its inhibition of antibody binding to toxin-coated wells (21). The amount of antibody bound to the wells is assayed by the amount of p-nitrophenyl absorbance generated after incubation.
of the wells with an alkaline phosphate conjugated to an anti-
mouse or anti-rabbit Ig and addition of
\( p \)-nitrophenyl phosphate. The more antibody bound to the toxin in solution, the
less binds to the wells, and the lower the absorbance value
measured.4

The degree of inhibition of antibody binding to ELISA plates
by toxin in solution was quantitated through the decrease in absorbance in the presence of toxin relative to the
controls lacking toxin.

The Effect of pH-induced Changes in Toxin Conformation on
Antibody Binding: Anti-C Domain Antibodies—Fig. 3
illustrates effect of the pH at which toxin is incubated upon anti-C
binding. \( \alpha\)-C1, \( \alpha\)-C2, and \( \alpha\)-C141-157 behave similarly in that
their binding is stronger to low pH-treated toxin, with a sharp
transition in binding near pH 5, the value at which the low
pH-induced change in toxin conformation has been shown to
occur (5, 6, 20). Antibody binding to toxin can be quantified by
the comparison of the concentration of toxin sufficient to inhibit
the ELISA color reaction by 50% (IC50) (Figs. 4,
A and B, and 5A). The IC50 values derived from these experiments are given
in Table I and show the difference in binding to low pH-treated
(Lr) and native toxin is greatest in the case of
\( \alpha\)-C1 (about 30-fold), but is also significant for the other anti-C
antibodies (about 2.5-fold).

The Effect of pH-induced Changes in Toxin Conformation on
Antibody Binding: Anti-T Domain Antibodies—Similar exper-
iments were performed with anti-T domain antibodies. Fig. 3B
illustrates the effect of pH at which toxin is incubated upon anti-T
binding. \( \alpha\)-T1, \( \alpha\)-T2, and \( \alpha\)-T224-237 each react differ-
ently with native toxin and low pH-treated toxin. \( \alpha\)-T1 shows
no significant dependence on the pH at which toxin was pre-
incubated. This is confirmed by the observation that IC50s for
native and low pH-treated (Lr) toxin binding by \( \alpha\)-T1 are the

4 The concentration of anti-toxins used were in a range that would
give a nearly linear color response with antibody concentration. Never-
theless, direct comparison of IC50 values cannot be used to compare the
strength of antibody binding for different antibodies because different
antibody concentrations were needed for each antibody to obtain suffi-
cient color reaction. Differences in color reaction may reflect different
binding of secondary antibodies to the primary antibody, or different
strength of binding of primary antibodies to the toxin-coated ELISA
wells.
Procedures).

Toxin (μg/ml) | Toxin (μg/ml)
---|---

**Fig. 5.** Assay of antipeptide antibody binding to the native (N) and low pH-treated (Lr') conformation of toxin using competition ELISA. Samples contained N (♦) or Lr' (♦) toxin. See Fig. 4 for details. Antibodies used were as follows: A, α-C141-157; B, α-T224-237.

**TABLE I**

| Antibody | Concentration (μg/ml) of toxin that yields 50% inhibition of ELISA reaction IC50<sup>a</sup> | Native (N) | Low pH-treated C domain folded (Lr') | Low pH-treated C domain unfolded (Lr') |
|---|---|---|---|---|
| Anti-C1 | 27.7 (30) | 0.94 (1) | 3.75 (4) |
| Anti-C2 | 0.53 (2.5) | 0.21 (1) | 0.82 (3.9) |
| Anti-C141-157 | 29.2 (2.3) | 12.6 (1) | 18.0 (1.4) |
| Anti-T1 | 1.05 (1) | 1.05 (1) | 9.25 (8.8) |
| Anti-T2 | 0.29 (0.15) | 1.89 (1) | 4.35 (2.3) |
| Anti-T224-237 | 16.4 (13) | 1.29 (1) | 2.60 (2.0) |
| Anti-R1 | 0.20 (0.21) | 0.95 (1) | 5.50 (5.8) |
| Anti-R2 | 1.26 (2.2) | 0.58 (1) | 4.00 (6.9) |

<sup>a</sup> Numbers in parentheses are IC<sub>50</sub> values normalized to 1 for toxin in the Lr' conformation.

The Effect of pH-induced Changes in Toxin Conformation on Antibody Binding: Binding to Isolated Domains—Binding of several antibodies to isolated domains was also measured. Iso- tated T and R domains appear to take on native and low pH-induced conformations similar to those they have in whole toxin (22, 23). Therefore, antibody binding to native and low pH-treated domains could be studied. The isolated domain binding of α-T and α-R antibodies that prefer binding the low pH-treated whole toxin was measured to see if the increased binding at low pH is due to increased exposure of their epitopes due to a loss of interdomain interactions, or to changes within the domain. Fig. 6 shows α-T224-237 binding to isolated T domain and α-R2 binding to the R domain have the prefer- ential binding to low pH-treated protein seen in whole toxin, supporting the latter proposal.

**DISCUSSION**

Using Monoclonal Antibodies to Evaluate Diphtheria Toxin Structure—Binding of anti-diphtheria toxin monoclonal antibodies to the toxin has previously been studied in other laboratories (27–33) which have found that monoclonal antibodies that bind to various sites on the toxin can be obtained. The studies of Zucker and Murphy (29, 30) tried to designate function to a particular area of the toxin by examining which functions were blocked by antibodies, but did not examine the effect of pH on toxin behavior.

A study by Rod and Eidel (33) did identify some mono- clonals that bind to low pH-treated toxin, but the number of pH values chosen for binding studies were insufficient to deter- mine whether the pH-dependent change in binding they ob- served corresponded to the conformational transition at pH 5.

This was confirmed for the R domain by the pH dependence of antibody binding and Trp emission. A similar low pH-induced conformational transition was observed with both these methods (data not shown).
Furthermore, the dependence of binding on pH was weak, perhaps reflecting the sensitivity of the immunoprecipitation method used.

In this study, antibodies to each domain of the toxin have been identified. These studies show that the binding of monoclonal and antipeptide antibodies can respond strongly to conformational changes in diphtheria toxin. Such antibodies should be useful tools for establishing whether toxin mutants fold and undergo conformational changes similar to those in whole toxin.

Just as important, these studies provide some new insights into the mechanism of diphtheria toxin insertion into membranes. One central observation in this study is that changes in all three domains occur at low pH. One obvious question is what changes are occurring in each of the domains? This is complicated to answer because several factors can influence antibody binding. A difference in antibody binding to two conformations could result from a difference in exposure of an epitope, or a change of an epitope into a conformation that is more weakly or tightly bound. Furthermore, a change in exposure of an epitope could be due to a change in the position or conformation of a sequence of a neighboring domain, or intermolecular aggregation.

Low pH-induced Changes in C, T, and R Domain Structure in the Lr′ Conformation—Despite these difficulties, the changes in antibody binding to toxin do provide some interesting details to what is occurring at low pH. Let us first consider the behavior of the toxin in the Lr′ conformation. Previous studies have shown that although there is some partial unfolding of the B chain in this conformation the C domain (A chain) remains folded (7). Therefore, the increased binding to low pH-treated toxin found for all three anti-C antibodies examined indicates there is increased exposure of the epitopes on the C domain to solution after low pH treatment. This suggests that the degree of contact between the C domain and the B chain (T plus R domains) must decrease at low pH. A related possibility is that the C domain undergoes a small conformational change that promotes antibody binding. In fact, a small conformational change in residues 66–78 and 169–176 has been seen in isolated C domain relative to whole toxin by crystallography (25), and is believed to be due to the loss of C domain interactions with the T domain. It is possible that our monoclonal antibodies bind C domain in whole toxin exposed to low pH more tightly due to a similar loss of C domain-T domain interactions. However, a more general increase in exposure at low pH is also very likely because the $\alpha$-C141–157, which does not involve the residues that change structure upon loss of T domain interaction, also binds more tightly at low pH. This conclusion is consistent with evidence that the interactions between the C and R domain are lost at low pH (34).

Additional changes occur in the T and R domain. The observation that the differences between the binding of some antibodies to these domains in the native and low pH-treated states are seen with isolated domains as well as whole toxin indicate that they are not solely due to changes in interdomain interactions, and probably reflect conformational changes within each of these domains at low pH. It is likely that there are conformational changes in these domains due to partial unfolding and exposing hydrophobic surfaces at low pH (12, 22). This conformational change would decrease the recognition to low pH-treated toxin of antibodies such as $\alpha$-R1 because the epitope would become hidden or change conformation. The same conformational change could cause increase antibody binding to low pH-treated toxin for antibodies such as $\alpha$-T224–237 and $\alpha$-R2 due to increased exposure or due to cooperative binding to low pH induced toxin oligomers (although see below).

Toxin Behavior in the Lr′ Conformation—For all of the antibodies tested, it appeared that binding to toxin in the Lr′ conformation was less than binding to the Lr′′ conformation. It is likely that in solution strong aggregation of the toxin in the Lr′ conformation generally decreases antibody binding to this conformation of the toxin. Previous studies have shown that the hydrophobic behavior of diphtheria toxin at low pH induces its aggregation in solution (6, 35). The observation that membrane insertion, which should eliminate the presence of aggregates held together by hydrophobic interactions, greatly increases antibody binding to the Lr′ conformation (see accompanying paper (39)) supports the proposal that aggregation in solution influences antibody binding. In contrast, aggregation does not seem to be a major influence on antibody binding to toxin in the Lr′ conformation because the antibody binding to Lr′ toxin in solution and inserted in model membranes is generally similar (compare Table I of the present
A model for the changes in diphtheria toxin at low pH and implications for membrane insertion—Based on the changes observed in diphtheria toxin at low pH, a schematic model summarizing the changes in toxin structure leading to membrane insertion can be proposed. The first stage would involve the loss of interactions between C and R domains, proposed by Bennett and Eisenberg (34). The next step would involve the major conformational changes observed in both the T and R domain, greatly increasing their hydrophobicity (Fig. 8). These conformational changes suggest that interactions between all three domains would break or become much weaker. This would also result in sites on all three domains becoming exposed to the aqueous environment after treatment with low pH, which could aid in membrane insertion. The unfolding of the C domain into a hydrophobic conformation, which requires slight more extreme conditions, may also occur at this time. Thus a number of coordinated changes may play a role in the membrane insertion process.

The observation of low pH-induced conformational changes in the R domain is important because it implies that the R domain plays an important role in insertion, rather than just functioning in binding to the cellular receptor (36–38). This is reinforced by the recent observation that the R domain becomes hydrophobic at low pH (23), and the results in the accompanying report, showing that the R domain inserts into membrane models in a fashion such that some sites translocate across the bilayer.

Comparison of Table I in this report and Table II in the accompanying paper (39) generally shows a similar IC50 for LR+ conformation toxin in solution compared to LR- toxin externally added to vesicles for several monoclonal antibodies. Comparison of results with and without lipid vesicles present is valid because the same monoclonal antibody concentrations were used in the solution and membrane-inserted toxin experiments, and other conditions were very similar except for a slight variation in salts used.

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