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The Conformation of Diphtheria Toxin: A Protein That Penetrates Membranes at Low pH

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There are two important reasons for considering the conformation of membrane-penetrating toxin proteins in the context of protein engineering. First, the "natural engineering" of such toxins, i.e., their conformation, involves some of the more fascinating designs found in nature, as they must undergo large conformational changes in order to function. Therefore, detailed study of toxin conformation will add appreciably to our understanding of the principles of protein folding. The second reason arises from the interest in artifical immunoglobulin–toxin covalent hybrids ("immunotoxins") as therapeutic agents targeted specifically against tumor cells. To design improved immunotoxin agents by protein engineering techniques it will be necessary to understand the mechanism of toxin action in detail.

I. DIPHTHERIA TOXIN STRUCTURE AND FUNCTION

The molecule we have been studying is diphtheria toxin. The toxin is a medium-sized protein (M_r 58,348) composed of two subunits: A (M_r

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21,167) and B (M_r 37199) (Greenfield *et al.*, 1983; Kaczorek *et al.*, 1983; Ratti *et al.*, 1983). The two subunits are held together by an exposed polypeptide link that is very sensitive to proteolysis. The sequence of the protein has certain noteworthy features. There are only two disulfide bonds, one within the B subunit and one connecting the A and B subunits. The protein has no free Cys residues. There are a total of five Trp residues scattered throughout the protein (two in A and three in B). This provides convenient intrinsic fluorescence probes at various regions in the molecule. There is a curious lack of His in the A subunit (there is only one residue, at position 21), while the B subunit has 15 residues. This may reflect the enzymatic function of the A subunit, which involves derivatization of a specific modified His, as described below.* Also important are several relatively hydrophobic strings of residues concentrated in the N-terminal half of the B subunit, likely candidates for membrane-penetrating regions (Greenfield *et al.*, 1983; Lambotte *et al.*, 1980).

The functionally important enzymatic reaction catalyzed by the A subunit is the transfer of ADP-ribose from NAD^+ to elongation factor 2 (EF-2), which thereby is inactivated. In this way the toxin shuts off protein synthesis. The ADP-ribose is attached to the amino acid diphthamide, a product of posttranslational modification of a His residue unique to EF-2 (Van Ness *et al.*, 1980). Other activities catalyzed by toxin are two apparent side reactions: (1) a slow NAD^+ glycohydrolase (NAD^+ nucleosidase) reaction, i.e., the splitting of NAD^+ into ADP-ribose and nicotinamide, and (2) self ADP-ribosylation. Interestingly, both the whole toxin and the A subunit have the NAD^+ glycohydrolase activity, suggesting that at least part of the A subunit is folded into its active conformation in the while toxin. However, the whole toxin does not catalyze ADP-ribosylation of EF-2, perhaps, as has been suggested, due to the B subunit sterically blocking the EF-2 binding site (Collier, 1982).

Considerable effort has been directed toward identifying the active sites and ligand binding sites. The NAD^+ binds to a site on the A subunit. The binding site includes Glu 148, as judged from photoaffinity cross-linking studies (Carroll and Collier, 1984). Isolated toxin is largely associated with dinucleotide ligands (80% ApUp, 15% ApGp) that bind extremely tightly, probably at this site (Barbieri *et al.*, 1981; Collins and Collier, 1984; Collins *et al.*, 1982). EF-2 evidently binds to the A subunit subsequent to NAD^+, but little else is known (Collier, 1982). Another site that has been identified is the so-called P site, which binds many anionic

* In fact, we find that the sequence Tyr-His-Gly-Thr around His 21 of subunit A is also found at His 440 of the ADP-ribosylating fragment of exotoxin A (Gray *et al.*, 1984) and there is some further limited homology on either side of the tetrapeptide. Therefore we suggest that it is possible that the tetrapeptide forms part of the active site.

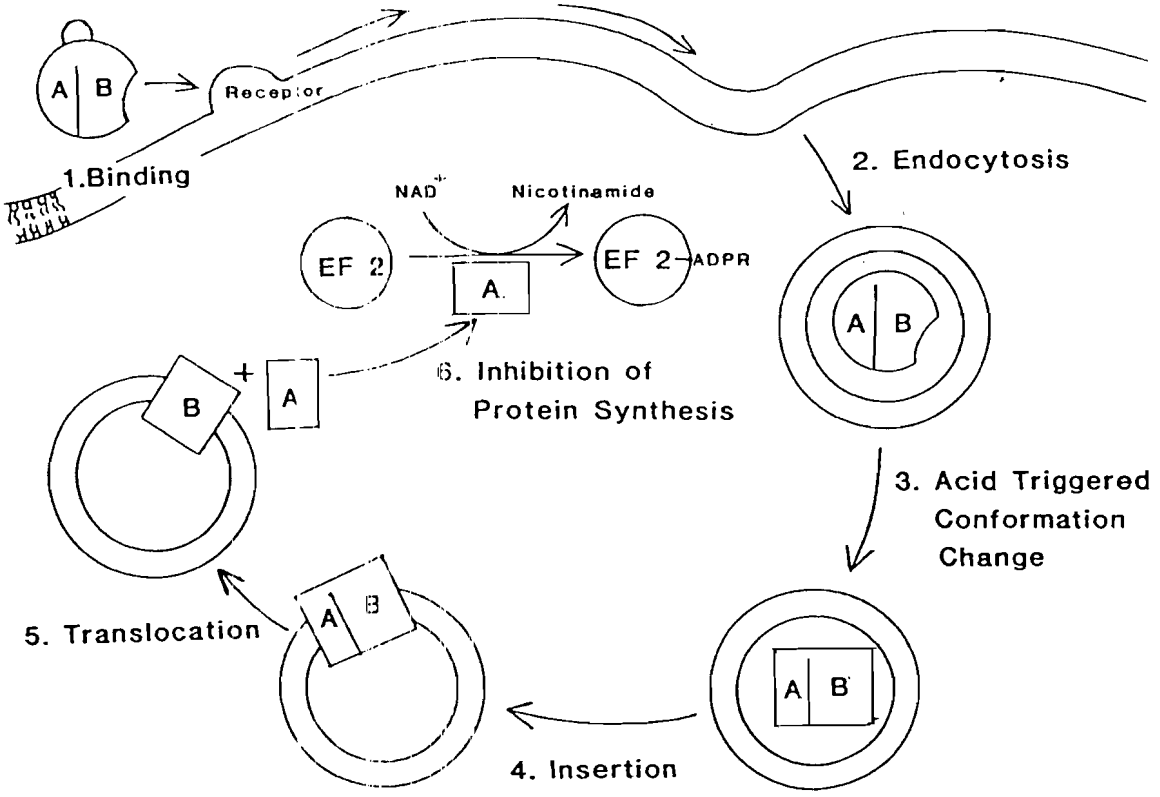


Fig. 1. Entry of diptheria toxin into cells; ADPR is ADP-ribose.

ligands (Collier, 1982). This site involves the C-terminal portion of the B subunit.

Spatially, this site is very likely to be adjacent to the NAD^+ site because of competitive inhibition between NAD^+ binding and P-site ligand binding (Collins and Collier, 1984). The C-terminal region of the B subunit also contains the binding site for the cell surface receptor, and the P and receptor sites may be closely related (Eidels *et al.*, 1982).

Figure 1 schematically illustrates the entry of diphtheria toxin into cells. Several studies now indicate that subsequent to receptor binding the toxin enters cells by receptor-mediated endocytosis. There has been much interest in the nature of the cell surface receptor. The bulk of the evidence supports the existence of a glycoprotein receptor for the toxin (Eidels *et al.*, 1983). However, based on the interaction with phosphorylated molecules, it has been suggested that certain lipids could act as receptors, although perhaps only of a secondary nature (Alving *et al.*, 1980). Endocytosis delivers the toxin to an acidic organelle (Sandvig and Olsnes, 1980; Draper and Simon, 1980), most likely an endosome (Marnell *et al.*, 1984), which is the name given to certain vesicles that are believed to be "intermediate" on the endocytosis pathway to lysosomes. The low pH in the lumen of these acidic organelles apparently triggers a hydrophilic-to-

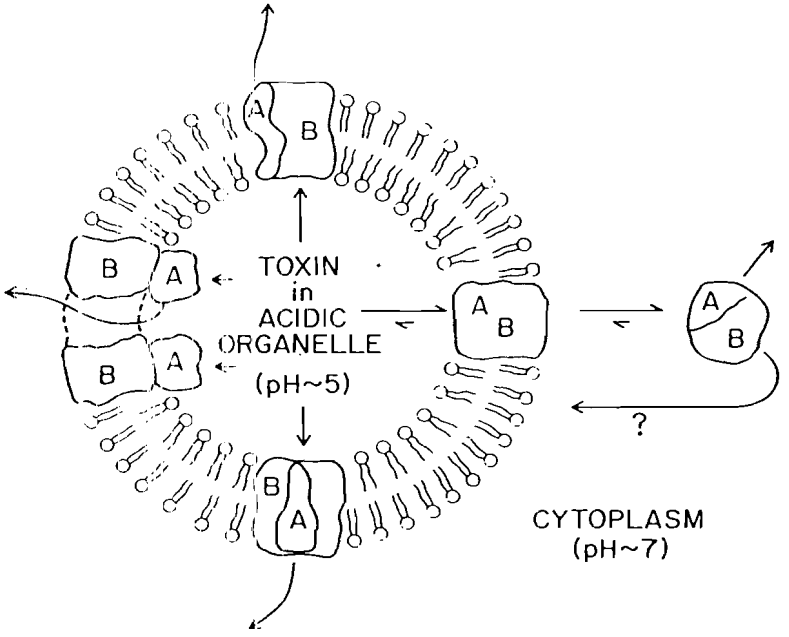


Fig. 2. Hypothetical mechanisms for membrane penetration.

hydrophobic change in toxin structure (Sandvig and Olsnes, 1981; Blewitt *et al.*, 1984, 1985). This is believed to result in insertion of the toxin into the lipid bilayer of the organelle membrane and, indeed, exposure to low pH results in insertion into model membranes *in vitro* (Donovan *et al.*, 1981; Kagan *et al.*, 1981; Zalman and Wisnieski, 1984; Hu and Holmes, 1984). In the next step the A subunit is released into the cytoplasm. It then turns off protein synthesis by the enzymatic ADP-ribosylation of EF-2 described above.

The least well-understood steps in diphtheria toxin entry involve the behavior of the toxin between the time of exposure to low pH and release of the A subunit. These steps require pronounced conformational changes. Already, several proposals have been made for the structure of membrane-inserted toxin, and these are shown in Fig. 2. Starting clockwise from the bottom, the first mechanism proposes that the toxin inserts such that the hydrophobic sites of the B subunit contact the bilayer and the hydrophilic A subunit is protected from contact with the bilayer. Upon release of the A subunit, a pore through the B subunit remains (Misler, 1984). In the second model this pore is formed by an oligomer of inserted B subunits. Indeed, toxin and isolated B subunit form pores in model membranes at low pH (Donovan *et al.*, 1981; Kagan *et al.*, 1981; Zalman and Wisnieski, 1984), but that does not prove they are important *in vivo*. One critical clue to their role is pore size. Any functional pore must be at least large enough to accomodate the A subunit in an unfolded

state. The reason subunit A need not remain folded in the membrane is that it can efficiently refold after exposure to strongly denaturing conditions (Collier, 1982). Unfortunately, studies attempting to determine the size of the pores have given different answers (Donovan *et al.*, 1981; Zalman and Wisnieski, 1984). Another possible model is that hydrophobicity of the toxin B subunit may be sufficient to "drag" the hydrophilic A subunit into the membrane so that it is in contact with, rather than shielded from, the bilayer. In fact, photocrosslinking studies have suggested that subunit A does contact the bilayer on insertion (Hu and Holmes, 1984; Zalman and Wisnieski 1984). The membrane-inserted A subunit would perhaps take on an unnatural conformation, but again, on release it could refold. Unfortunately, the presence of reactive amino acid residues can distort cross-linking results even when "nonspecific" reagents are used (Ross *et al.*, 1982), so the exact degree of exposure of A subunit to the bilayer is uncertain. Another possible mechanism involves translocation of the whole toxin. If the pH-sensitive sites which trigger a change to a hydrophobic conformation at low pH became exposed to the neutral pH cytoplasm after insertion, then the toxin might switch back into the hydrophilic conformation and dissolve in the cytoplasm. In fact, starting at pH 3, which avoids excessive aggregation (Blewitt *et al.*, 1985), low-pH conformational changes are mostly reversed on neutralizing pH. However, this model assumes that the sites controlling the conformational transition become exposed to the cytoplasm upon insertion, and there is no supporting evidence for this assumption. A final possibility, not shown in Fig. 2, is that the whole toxin breaks endosomes open, resulting in release of the toxin. Indeed, recent studies showing that toxin can induce vesicle fusion at low pH suggest that the toxin has the potential to disrupt membranes (Cabiaux *et al.*, 1984), and it has been proposed that toxin enters the cytoplasm in a burst involving a number of toxin molecules (Hudson and Neville, 1985). At present we cannot say which model is correct.

Once the A subunit is exposed to the cytoplasm it can presumably be released from the B subunit if the disulfide bond between the subunits has been cleaved by reduction. Reduction of disulfide bonds by natural sulfhydryl agents such as glutathione is unlikely to take place in the lumen of an acidic organelle. The low pH of the lumen in an acidic organelle will prevent reduction because such reactions depend on the S^- form of the thiol (Torchinsky, 1981). Instead, previously proposed models assume that this cleavage occurs in the reducing environment of the cytoplasm, which places reduction subsequent to insertion, although it has also been suggested that membrane proteins could interact with toxin disulfides (Wright *et al.*, 1984).

II. THE HYDROPHILIC-TO-HYDROPHOBIC SWITCH: TRANSITION pH

The preceding discussion demonstrates that it will be difficult to characterize the true mechanism by which the toxin penetrates membranes and accomplishes translocation of the A subunit. Our approach is to use biochemical and biophysical techniques to dissect each step. Our first studies have concentrated on the nature of the switch to a hydrophobic conformation (Blewitt *et al.*, 1984, 1985). The experiments were done with 10^{-7} *M* toxin. This concentration is close to the physiological concentration expected in acidic organelles because the concentration of a single toxin molecule in a spherical acidic organelle of diameter 0.1–1.0 μm would be 3×10^{-6} to 3×10^{-9} *M*, respectively, as calculated from the number of moles of toxin divided by the internal volume. It may even be that this is an underestimate of concentrations *in vivo* because the concentration of toxin molecules may be higher than one per organelle (Hudson and Neville 1985).

Our observations revealed that the change induced by low pH, and detected by Trp fluorescence intensity, is a highly cooperative transition, which occurs over a pH range of only 0.2 unit (Fig. 3). The midpoint, at 23°C, is close to pH 5. This falls within the range of pH encountered in acidic organelles, about pH 4.8 in lysosomes (Geisow, 1984), and pH 5–5.5 in endosomes (Maxfield, 1982; Geisow and Evans, 1984), and therefore suggests that the transition observed *in vitro* could be the same as that which occurs *in vivo*. This conclusion is reinforced by the kinetics of the transition, which is very fast at pH 3 or 4.2 ($t_{1/2} < 30$ sec). This is important as the time for cytotoxicity (i.e., EF-2 inactivation) to appear is on the order of minutes at high toxin concentrations, and therefore any changes requiring a long time are unlikely to be physiologically significant.

Several environmental conditions and structural variations may affect the transition pH. Ionic strength is one such factor. In low salt concentrations the transition shifts to pH 4, while in the presence of 150 m*M* monovalent salts the transition pH is consistently 5. This sensitivity to ionic strength is an important clue to the mechanism of the transition, as discussed later. On the other hand, the transition pH is not influenced by the form of toxin used. Bound toxin (which contains one molecule of tightly bound dinucleotide), free toxin, toxin nicked between A and B or with an intact polypeptide, monomer, or dimer, all have very similar pH transitions. One factor we have not been able to test is the effect of toxin binding to receptor on the transition pH. However, the studies of Draper and Simon (1980) indicate that receptor binding does not have a major effect. They found that receptor-bound toxin directly penetrated the

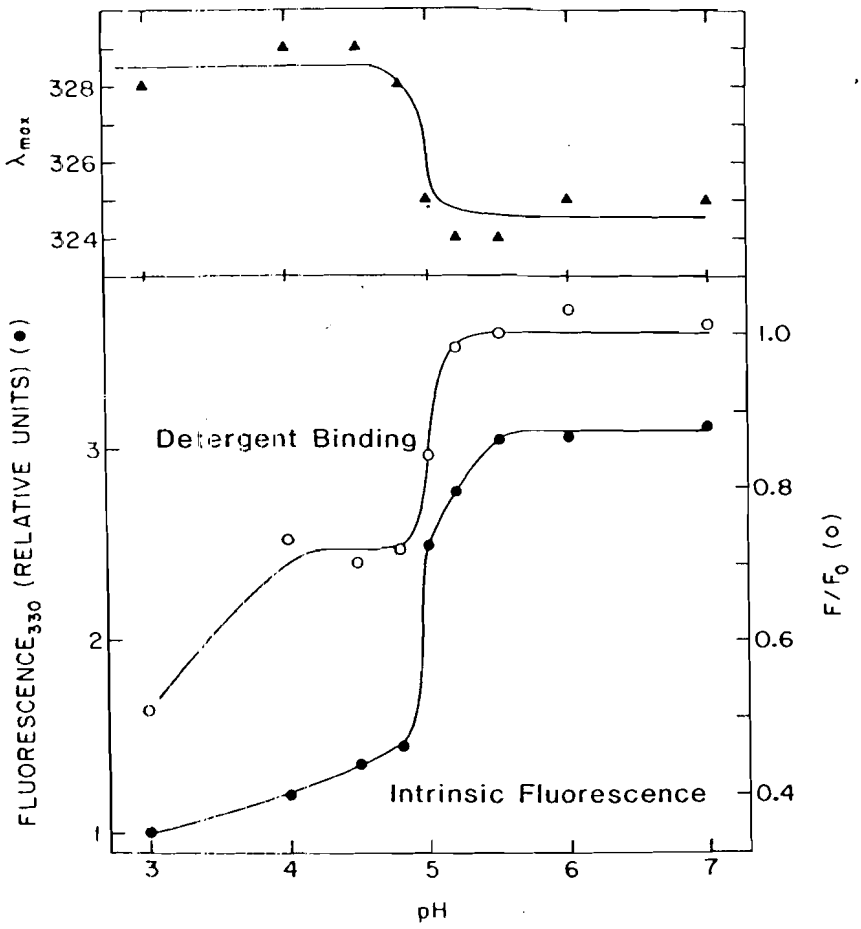


Fig. 3. Effect of pH on toxin fluorescence and detergent binding. F/F_0, Ratio of fluorescence in the presence of quenching detergent micelles to that in the presence of micelles without quencher. An F/F_0 value less than 1 indicated detergent binding. (Adapted from Blewitt *et al.*, 1985. Copyright 1985, American Chemical Society. Reprinted with permission.)

plasma membrane when cells were incubated below pH 5. The implication is that below pH 5 the receptor-associated toxin becomes hydrophobic. Since this is the same pH at which "receptor-free" toxin becomes hydrophobic, one must assume that receptor binding is not a critical regulatory factor. It is not even clear if the toxin remains receptor-bound at low pH.

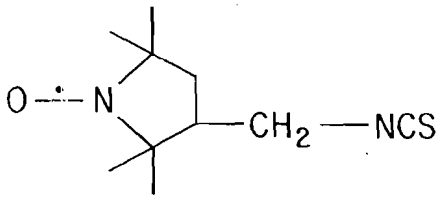
III. THE HYDROPHILIC-TO-HYDROPHOBIC SWITCH: CONFORMATIONAL CHANGES

Below the transition pH the toxin undergoes several distinct changes in physical properties which we have tried to characterize. Most important of these is the dramatic increase in hydrophobicity. We have developed new fluorescence quenching methods that measure this hydrophobicity

through binding to micelles of mild nonionic detergents. In this assay the intrinsic protein (Trp) fluorescence intensity is measured in two samples, one of which contains ordinary micelles and the second contains micelles with fluorescence quenchers. If the protein is hydrophobic it will bind to the micelles and weaker fluorescence will be observed in the sample with quencher. This method considerably simplifies the measurement of hydrophobicity. As seen in Fig. 3, there is considerable detergent binding by toxin only below the transition pH. Binding studies show that binding of 10^{-7} *M* toxin to micelles of the detergent Brij 96 (critical micelle concentration, 3 μM), saturates at 15 μM detergent. An important question is whether this binding is tight enough to explain spontaneous insertion of organelle-trapped toxin molecules into the organellar membrane. Calculations suggest that this is so. If we assume again that toxin is trapped in an organelle of diameter 0.1–1.0 μm, then the apparent lipid concentration will be given by the moles of lipid facing the interior divided by the internal aqueous lumen volume. The moles of lipid can be calculated in turn from the internal surface area of the organelle membrane divided by the number of lipids per unit area. Assuming 70 $Å^2$ per lipid molecule, and that 50% of the surface is occupied by protein and 50% by lipid, gives a 7–70 m*M* apparent lipid concentration for a 1.0- or 0.1-μm-diameter organelle, respectively. This far exceeds the concentration necessary to observe tight binding *in vitro*.

Other changes in the structure of the toxin accompany the increase in hydrophobicity. There is an increase in average exposure of Trp residues to the solution, as judged both from a red shift in λ_{max} and an increase in acrylamide quenching at low pH. Circular dichroism (CD) shows a change in secondary structure at low pH. At neutral pH the protein appears to be rich in β sheets by CD although considerable α-helix may also be present (Blewitt *et al.*, 1985; Collins and Collier, 1985). The changes observed in CD at low pH must likely involve a slight increase in random coil.

These changes in secondary and tertiary structure could be interpreted as a partial denaturation at low pH. Support for this concept comes from the effect of high temperature on the conformation of the toxin and its transition pH (Zhao and London, 1985). In studies of the thermal denaturation transition we found that the thermally denatured conformation resembles the low-pH conformation in several respects, although they are not identical. In particular, both conformations show increased Trp exposure and hydrophobicity. Furthermore, as pH is decreased the thermal transition temperature is decreased, and as temperature is increased the transition pH increases. This implies that at least some of the interactions in the toxin that can be disrupted by thermal denaturation at high temperature are similarly disrupted at low pH. Therefore, the conformational



(I)

change at low pH can indeed by thought of in terms of partial denaturation. On the other hand, it must be emphasized that extensive unfolding of the polypeptide does not occur at low pH. The conformation of the toxin in guanidinium Cl or urea is clearly distinct from that at low pH or at high temperature. Unfolding may involve only a very limited domain at low pH or reflect the loss of interactions between domains.

These conclusions are supported by electron spin resonance (ESR) studies of dipththeria toxin spin-labeled at a 2 : 1 (mole/mole), ratio with the amino specific isothiocyanate probe (**I**).

At pH 7 the ESR signal of the labeled toxin indicates that there are two different spin-label environments that fall in the weakly to moderately "immobilized" range. A single isotropic mobile signal is found under completely unfolded conditions, such as in urea or guanidinium Cl, but not at low pH or after denaturation by high temperature. Unfortunately, the ESR signal appears to be sensitive to aggregation, which is extensive both at low pH and high temperature (Blewitt *et al.*, 1985; Zhao and London, 1986), and thus a more precise analysis of conformation from ESR spectra will be complex.

The finding of aggregation is important because it can complicate experimental interpretation in several types of experiments. For example, aggregation via contacts between hydrophobic sites can compete with detergent binding, reducing the amount of bound detergent and thus the apparent hydrophobicity judged from detergent binding. Aggregation of the low pH conformation could also affect the balance between the neutral and low pH conformations (and thus the apparent transition pH) by driving the reaction toward the low pH conformation. It could affect the degree of the cooperative dependence upon proton concentration as well. Also, CD spectra can be distorted by aggregation. Perhaps most important, if nonphysiological aggregates occur when toxin is inserted into model membranes, then serious artifacts may result. On the other hand, one cannot rule out a functional role for aggregation. Studies showing that a single A subunit artifically introduced into the cytoplasm can eventually kill a cell (Yamaizumi *et al.*, 1978) do not prove that toxin penetrates membranes in monomeric form. In any case, the influence of aggregation must be a careful consideration in future studies.

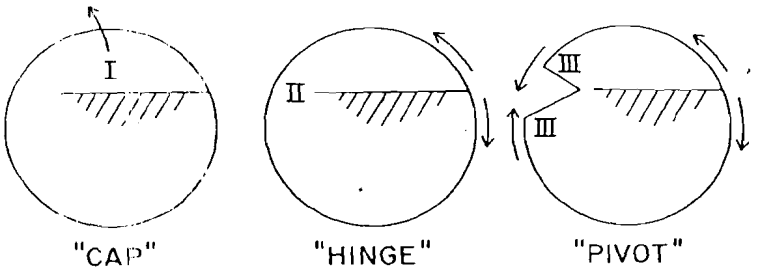


Fig. 4. Possible nature of conformational changes at low pH. Hatched area represents hydrophobic sites. Numerals show region of conformational change.

IV. MECHANISM OF THE CONFORMATIONAL CHANGES

On the basis of these results one can ask, What specific changes in toxin structure occur at low pH? Some possible models are shown in Fig. 4. First, a domain covering the hydrophobic site and buried Trp residues might unfold, thereby exposing buried sites. Second, a hinge holding two domains together could unfold, breaking interdomain interactions, causing domains to come apart, and thereby exposing buried sites. Such a mechanism has been proposed for the change undergone by staphylococcal α-toxin in the presence of lipids or detergents (Tobles *et al.*, 1985). Third, one could imagine a "pivoting" mechanism in which the change in conformation involves formation of intrapolypeptide interactions. For example, in the Bohr effect on hemoglobin conformation, protonation stabilizes certain bonding interactions while weakening the interaction with oxygen. However, the denaturation-like effects of low pH are more consistent with the first two models. Fourth, low pH could create a hydrophobic surface directly without a conformational change by protonation of surface Asp and Glu residues, as proposed recently for colicin E_1 (Davidson *et al.*, 1985). This must be ruled out in view of the evidence for a preformed buried hydrophobic site in the sequence data and in experiments with mutant toxins (Boquet *et al.*, 1976), evidence for large conformational changes and, as explained below, because of the effect of salt on the transition pH. As noted earlier, increasing ionic strength by addition of 150 m*M* salt increases the pH of the hydrophilic-to-hydrophobic transition by one unit, but salt shifts the pK_a values of Asp and Glu to lower pH, which means that this fourth mechanism predicts a decrease of the transition pH, opposite to the observed effect. Nevertheless, even though it cannot be the only change occurring at low pH, an increase in hydrophobicity due to charge neutralization could still be one component of the transition.

A second question is, What causes the change in conformation at low

pH? Clearly, protonation of amino acid residues must be of central importance. If we let N represent the native conformation, which predominates at neutral pH, and L the conformation which predominates at low pH, then the binding of protons must affect the equilibrium between N and L states such that in the protonated state L predominates. The question can be restated as: How does protonation raise the energy of the N conformation relative to the L conformation?* To answer this question we assume, based on the conclusions above, that the L conformation shows increased exposure of buried groups and has unfolded, at least to the degree that its structure is less compact than in the N state. In this case, the changes in electrostatic interactions induced by protonation will affect the energy of the two states differently.† There are a number of ways in which stabilizing electrostatic interactions might be lost, and destabilizing ones formed, on protonation of acidic or basic amino acid residues. For example, protonation of buried acidic residues could break "stabilizing" internal salt bridges. Protonation of basic residues near other positively charged residues could give rise to electrostatic repulsions. Alternatively, if a basic residue is deeply buried in a hydrophobic site, an energetically unfavorable isolated charge could form on protonation. Such energetically unfavorable changes would tend to destabilize the N state more than the L state because the L state would tend to have an increased distance between charges and would tend to have more charges exposed to solvent and thus in a more polar environment, both of which would diminish the influence of electrostatic effects. Therefore, the equilibrium would shift toward the L state upon protonation. It should be noted that it is difficult to precisely identify the types of ionizable groups involved directly from the apparent pK_a of the transition, because it is a complex function of the pK_a values of all involved residues, both in the N and L states (Tanford, 1970). For example, although the apparent pK_a of the transition is below the normal pK_a for isolated, exposed basic residues, the participation of basic groups is *not* at all ruled out because they would have lower pK_a values when buried in the N state as described above.

The sensitivity of the transition to ionic strength suggests the importance of electrostatic interactions. As noted earlier, the transition pH shifts from pH 4 to 5 in 150 m*M* NaCl. Ionic strength has an effect on pK_a, decreasing the pK_a of acids and increasing the pK_a of bases. Therefore, one explanation for the salt-induced shift to increasing pH could be that

* Alternatively, because the conformational and ionization equilibria are coupled, it is equally valid to formulate the question as: Why does L bind protons more strongly than N?

† Of course, upon protonation there can also be some changes in hydrogen bonding, hydrophobicity, and Van der Waals interactions.

protonation of basic residues triggers the transition. However, the pH shift observed is much larger than the expected shift in pK_a (Perrin and Dempsey, 1974), and therefore other factors may be involved. Indeed, a further effect would arise from electrostatic repulsions between like-charged sites. The presence of such sites would also shift the equilibrium toward the L state in salt, because the stabilizing shielding of electrostatic repulsions by counterions would be more effective in the less compact L conformation, allowing more effective penetration of counterions from solution (Tanford, 1961). Another effect of increased ionic strength would be to weaken intramolecular salt bridges by competition from ions in solution, so again salt bridges could be important.

Examination of the amino acid sequence of the B subunit hints at the sites of possible electrostatic interactions. Figure 5 shows a plot of a parameter we have defined as the excess charge fraction (ECF). The ECF is simply the number of net formal excess positive or negative charges in a segment divided by the total number of charges in the segment. For this calculation we assign a charge of −1 for Glu and Asp and +1 for Arg, Lys, and His. The ECF shows that, as previously known, the C-terminal region of the B fragment has excess positive charge. Intriguingly, the N-terminal half of the B fragment has a large negative ECF. This represents not a large number of negative charges, but rather the fact that the few charged residues among the hydrophobic stretches tend to be negatively charged residues. One possibility suggested by this is that the C-terminal region folds over the N-terminal region so that critically important salt bridges form between the two. Indeed, studies with a mutant toxin have demonstrated that deletion of the C-terminal does expose hydrophobic sites (Boquet *et al.*, 1976). On the other hand, one can easily propose alternative interpretations. One possibility is that the acidic residues are not involved in salt bridges, but instead function in pH-dependent regulation of the degree of hydrophobicity of the N-terminal half. As for the C-terminal region, perhaps it unfolds due to increased electrostatic repulsions as its basic residues become protonated. Another possibility is that the excess positive charge in the C-terminal region is involved only in the previously identified receptor and/or polyanion binding sites and has no role in the transition. Even explanations of the transition mechanism in which electrostatic interactions have no role can be conceived. For example, it has been suggested that proline isomerization catalyzed by low pH could be important (Deleers *et al.*, 1983). Clearly, all such hypotheses are pure speculation at this point and will remain so until the crystal structure, now being determined by two groups of investigators, is available (Collier *et al.*, 1982; McKeever and Sarma, 1982). We must also caution that at present it is not really known whether the conformational changes

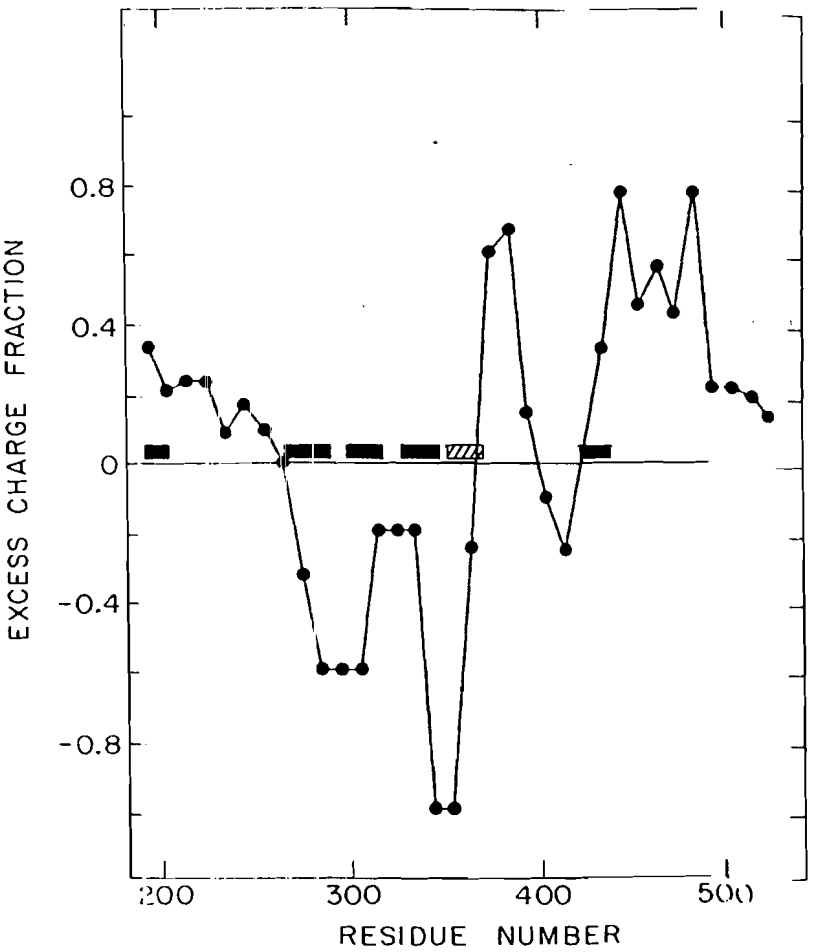


Fig. 5. Charge profile of B subunit. Stretches of 10 or more uncharged amino acid residues are shown as solid blocks. Lined block has only one charged residue. Data points are excess charge fraction (see text) calculated for a 30-residue block; point is placed at center residue. Residue number corresponds to position in overall polypeptide sequence (Greenfield *et al.*, 1983).

occur only within the B subunit, A subunit, the A–B interface, or a combination of these sites. It is very likely that the cooperative transition involves a number of different changes.

V. IMPLICATIONS FOR THE CONFORMATION OF OTHER PROTEINS AND DESIGN OF MODIFIED TOXINS

An important implication of these studies is the possibility that the design of diphtheria toxin conformation may be very similar to that of other proteins believed to undergo a conformational change triggered by low pH in acidic organelles. One of the best characterized cases is that of

the influenza virus hemagglutinin, which is believed to play a critical role in low-pH fusion of viral envelope with the membrane of an acidic organelle (White *et al.*, 1983). The pH needed for influenza virus fusion is very similar to the transition pH for the toxin. Furthermore, like the toxin's transition, fusion occurs rapidly on exposure to low pH, and the switch between fusogenic and nonfusogenic states occurs over a narrow pH range. In addition, salt bridges in the hemagglutinin have already been implicated in the pH-induced conformational change (Wilson *et al.*, 1981; Daniels *et al.*, 1985), and a possible relationship between the effects of high temperature and low pH has been hinted at (Daniels *et al.*, 1985). Similar behavior at low pH has also been proposed for a number of other viruses (White *et al.*, 1983) and toxins (Hoch *et al.*, 1985). Furthermore, it is likely that low pH regulates the dissociation of some receptor–ligand complexes at low pH (Ciechanover *et al.*, 1983). So understanding the behavior of the toxin may help us understand the behavior of a whole class of proteins and vice versa. For example, the similarity between influenza hemagglutinin and toxins may help in the design of therapeutic agents. Amantadine, which is used prophylactically and therapeutically for influenza, probably acts as a lysosomotropic agent, increasing the pH in acidic organelles. It might be useful in treating diphtheria as well by preventing membrane penetration by the toxin.

What lessons do these studies have for the design of modified toxins? First, addition or removal of salt bridges may be a key to altering the stability of the protein. Second, basic as well as acidic residues may participate in acid triggering of a conformational change. Finally, any changes that affect thermal stability can also affect pH stability, even if they do not involve ionizable residues. From these ideas it seems that use of oversimplified models of protein structure to design specific changes will give unsatisfactory results in some cases, because the critical residues to change will not always be apparent just from the sequence. Of course, without the high-resolution X-ray structure and characterization of a series of single site mutants, these conclusions can only be a list of possibilities rather than a firm set of rules. Nevertheless, they serve as a starting point for the design of further experiments and suggest that when a combined approach to the structure of these molecules is pursued we will be able to use the understanding gained to design useful modified toxin proteins.

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