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Similarity of the conformation of diphtheria toxin at high temperature to that in the membrane-penetrating low-pH state

(denaturation/fluorescence/detergent binding)

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ABSTRACT **At high temperature, nicked free monomers of diphtheria toxin undergo a transition to a thermally denatured state, with a midpoint of 45–50°C. In this report, the high-temperature (60°C) conformation has been compared to the native (neutral pH) and low-pH (pH < 5) conformations. The low-pH and high-temperature conformations are similar although not identical. As in the conformation at low pH, and unlike the toxin in its native conformation, the protein in its conformation at high temperature is hydrophobic, has low fluorescence intensity, and has increased exposure of tryptophan to aqueous solution. As at low pH, at high temperature the circular dichroism spectrum shows at most only partial unfolding of secondary structure. In contrast, the conformation of the toxin in guanidinium chloride is much closer to a random coil. The effects of high temperature and low pH interact in the sense that sensitivity of the native conformation to one is increased by the other. That is, the transition temperature between native and thermally denatured states is decreased as pH is decreased, and the transition pH between neutral-pH and low-pH states is increased as temperature is increased. This implies that there is some region on the protein where high temperature and low pH can disrupt conformation in a similar manner. Taken together, these results indicate that the low-pH and high-temperature conformations can both be defined as partially denatured states, even though unfolding may not be extensive at low pH. Similar behavior may occur in other proteins that undergo functionally important conformational disruption at low pH.**

Diphtheria toxin is a protein (M_r = 58,340) that kills cells by inhibition of protein synthesis. It is composed of two domains: subunit A, which inhibits elongation factor 2 by ADP-ribosylation, and subunit B, which binds to a receptor molecule and is required to translocate subunit A into the cytoplasm. The amino acid sequence of the toxin is known (1–3) and its enzymatic function has been extensively studied (4–6). Several lines of evidence indicate that toxin enters cells by receptor-mediated endocytosis, followed by penetration through the membrane of an acidic organelle (7–10). Recent studies have started to characterize toxin behavior at low pH (11–16). Nevertheless, the details of toxin conformation upon membrane penetration and the mechanism of subunit A translocation remain unknown. Elucidation of the mechanism of membrane penetration by toxin may have important implications for designing immunotoxins (17), for understanding the viral fusion proteins that control viral penetration through acidic organelles (18), and for insertion and translocation of newly synthesized membrane proteins.

In previous studies we have characterized the native and low-pH conformations of the toxin (19, 20). In this report we compare the conformational changes at high temperature and in denaturant to those at low pH. The low-pH and high-temperature conformations seem to be closely related.

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MATERIALS AND METHODS

Free [i.e., without bound ApUp (21)] monomers of diphtheria toxin were isolated and nicked proteolytically as described previously (19, 22). Acrylamide (electrophoresis grade) was purchased from Bio-Rad. Guanidinium chloride (GdmCl) and Brij 96 were purchased from Sigma. Brominated Brij 96 was prepared as previously described (19, 20). 1,2-dipalmitoyl-*sn*-glycero-3-phospho[*N*-(1-pyrenesulfonyl)]ethanolamine (DPPE-pyrene) was purchased from Molecular Probes (Junction City, OR). A stock solution of mixed micelles of 0.5% DPPE-pyrene/0.025% brominated Brij 96 was prepared by 30-min sonication (bath sonicator, Lab Supplies, Hicksville, NY) of DPPE-pyrene in water. Then brominated Brij 96 was added and the mixture was incubated at 50°C to help dissolve the lipid. It was stored at 23°C.

Fluorescence was measured with a Spex 212 spectrofluorimeter as previously described (20). CD was measured on a Cary 60 spectrophotometer with a 6001 CD attachment. CD was calibrated as previously described (20). Protein was assayed by A_{280} as described (19, 20).

Samples prepared at low pH, high temperature, or with GdmCl were incubated 30 min before taking measurements. To measure the temperature dependence of toxin properties, temperature was first increased and then decreased. Temperature was recorded at the time of each measurement with a digital probe thermometer. Measurements were taken every 5–10 min, subsequent to temperature stabilization. Under these conditions the incubation time dependence of the changes in fluorescence was relatively small.

RESULTS

The thermal transition in nicked, free monomers of diphtheria toxin is shown in Fig. 1 *Left*. The midpoint of the transition is 48°C as detected by fluorescence intensity.† The transition temperature in dimer toxin is 51°C as judged by fluorescence intensity (data not shown). These values fall within the temperature range previously found by scanning calorimetry for toxin unfractioned by size (23). Above the transition there are irreversible changes similar to those observed at low pH (19, 20), including a decrease in fluorescence intensity, a red shift in λ_{max} of tryptophan emission, and the appearance of hydrophobicity, as judged by fluorescence quenching. This

Abbreviations: GdmCl, guanidinium chloride; DPPE-pyrene, 1,2-dipalmitoyl-*sn*-glycero-3-phospho[*N*-(1-pyrenesulfonyl)]ethanolamine.

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†Average fluorescence λ_{max} is weighted both by the fluorescence intensity and fraction of each conformation present. Therefore, λ_{max} overrepresents the amount of native conformation present, resulting in a slightly higher apparent transition midpoint.

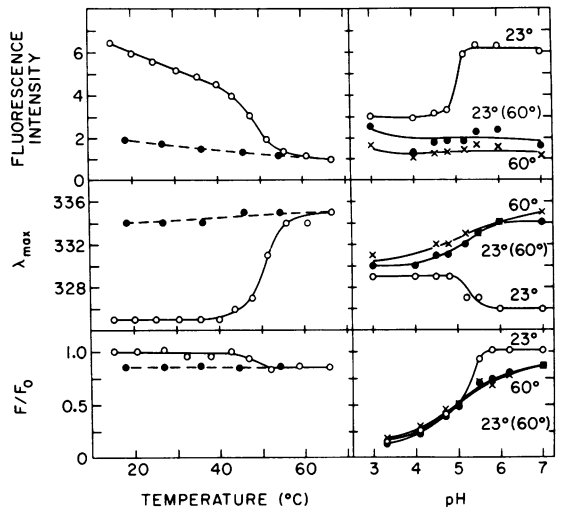

FIG. 1. Effect of temperature and pH upon toxin conformation. (*Top*) Fluorescence intensity. $\lambda_{excitation}$ = 280 nm; $\lambda_{emission}$ = 325 nm. (*Middle*) λ_{max} (nm) of fluorescence emission. (*Bottom*) Hydrophobicity detected by detergent binding. Ratio of fluorescence intensity (F) in the presence of quenching micelles (0.0005% DPPE-pyrene/0.01% brominated Brij 96) to fluorescence intensity (F_0) in the presence of nonquenching micelles (0.01% Brij 96) was measured. $\lambda_{excitation}$ = 295 nm; $\lambda_{emission}$ = 335 nm. A ratio less than 1 indicates detergent binding (see text). (*Left*) Effect of increasing (○) and then decreasing (●) temperature. Samples contained toxin (nicked, free monomers) at 10 μg/ml in 150 mM NaCl/10 mM P_i, pH 7. (*Right*) Effect of temperature upon pH dependence of toxin properties. ○, 23°C; ×, 60°C; ●, 23°C after incubation at 60°C for 30 min. Samples contained toxin at 10 μg/ml in 10 mM buffer/150 mM NaCl. Buffers used were formate (3 < pH < 4); acetate (4 < pH < 5.5), and P_i (pH > 6.0).

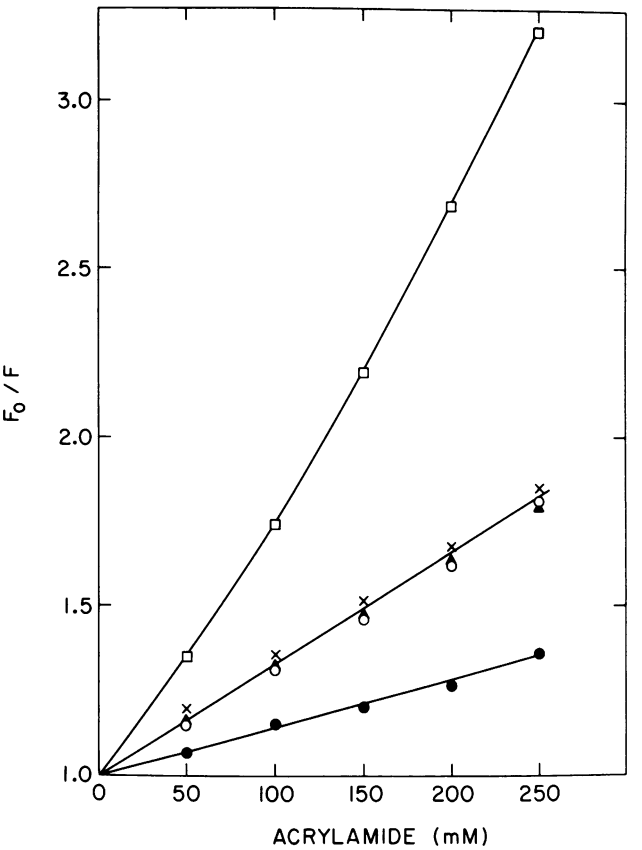

FIG. 2. Acrylamide quenching of fluorescence in different toxin conformations. Samples contained toxin at 10 μg/ml in 150 mM NaCl/10 mM Tris·HCl, pH 7, unless otherwise noted. ●, Native toxin; ○, toxin incubated at 60°C, quenching measured at 23°C; ×, toxin in 10 mM sodium formate/150 mM NaCl, pH 3; ▲, toxin in pH 3 buffer at 23°C after incubation at 60°C; □, toxin in pH 7 buffer/NaCl/3 M GdmCl.

fluorescence quenching assay detects binding to micelles of a mild detergent as a decreased fluorescence in the presence of micelles containing a quenching probe relative to that in micelles without the probe (i.e., a decrease in F/F_0) (19, 20). Also observed is a decrease in fluorescence intensity outside the transition range of about 1.2–1.3%/°C. This is due to ordinary reversible reduction of quantum yield by thermal effects, not a conformational change (24).

Fig. 1 *Right* shows the effect of pH on toxin conformation at 23°C and at high temperature (60°C). A pH transition in fluorescence intensity is observed at 23°C, as characterized in previous studies (19, 20). At 60°C the transition is replaced by a more gradual change. At 23°C, λ_{max} undergoes a red shift at low pH as noted previously (19, 20). At 60°C a much more gradual *blue* shift occurs at lower pH. At 23°C, quenching (and therefore detergent binding) occurs only below the transition pH, as expected from previous studies (19, 20). At 60°C a more gradual increase in quenching is observed as pH decreases. These properties show that the low-pH and high-temperature conformations are not identical despite their similarity. Also, Fig. 1 *Right* shows that the changes in behavior at 60°C are not reversed at 23°C, with the trivial exception of the thermal reduction in fluorescence intensity noted above.

The fluorescence properties of toxin in 3 M GdmCl show similarities to those at low pH and high temperature. This includes a severalfold decrease in fluorescence intensity (midpoint concentrations ≈ 0.5–1 M), and a large red shift in λ_{max} of emission to 347–348 nm, close to the value for free tryptophan (350–352 nm) (data not shown).

Fig. 2 shows further characterization of these conformations by using acrylamide quenching. Acrylamide quenching measures the degree of exposure of tryptophan to the aqueous medium (25, 26). Average exposure is roughly proportional to the slope of the quenching curves, provided fluorescence lifetimes are not very different in different conformations. The curves show that the tryptophan residues are relatively buried in the native state and exposed in GdmCl. In fact, quenching of free tryptophan is very similar to that of tryptophan residues of toxin dissolved in GdmCl (not shown). Exposure is about equal at low pH or after high-temperature incubation, being intermediate between the exposures in the native and GdmCl conformations in these cases. Possible lifetime variations are insufficient to explain these differences (20, 25).

Fig. 3 shows quenching-detected detergent binding curves for the various conformations. Detergent binding in the low-pH, high-temperature, and GdmCl conformations is very tight, with binding half-maximal between 0.0002% and 0.0005% detergent (2.8–6.9 μM). Therefore, all three states must be very hydrophobic. However, the final levels of quenching are very different. This is probably due to two factors. First, quenching is dependent on conformation because conformation affects tryptophan–quencher distance. When the separation is large quenching will be weak. This probably explains the weak quenching in GdmCl, in which λ_{max} remains at 348 nm in detergent and thus the tryptophan residues are largely in contact with water rather than quenching micelles. Second, protein aggregation (20) will reduce quenching, because protein–protein contacts at hydrophobic sites will replace some of the bound quencher. Since toxin aggregation is more extensive at high temperature than at low pH, and since detergent binding does not completely break up aggregation (unpublished observations), this probably explains the weaker quenching observed at 60°C.

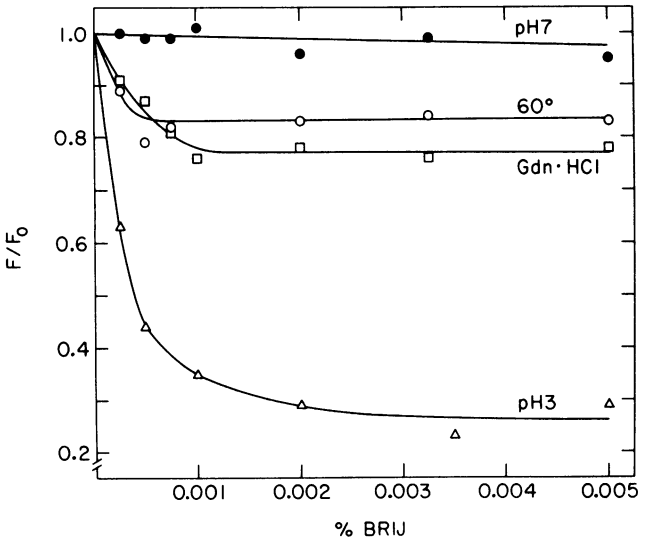

FIG. 3. Binding of toxin to micelles. Samples contained toxin at 5 μg/ml in 150 mM NaCl/10 mM P_i, pH 7, unless otherwise noted. Separate toxin samples were made at each detergent concentration and incubated for 30 min before the ratio of fluorescence in the presence and absence of quenchers was determined. ●, Native toxin; ○, high-temperature conformation (measured at 60°C); △, low-pH conformation in 150 mM NaCl/10 mM formate, pH 3; □, toxin in pH 7 buffer/NaCl/and 3 M GdmCl.

Fig. 4 shows the CD spectra for the various toxin conformations. The spectra of the native and low-pH conformations have been described previously. CD in this range is sensitive to changes in secondary structure. The negative ellipticity observed in the low-pH and high-temperature conformations suggest they have considerable secondary structure. Unfortunately, aggregation distorts CD spectra (27), so it is not possible to analyze the high-temperature CD spectrum in more detail. Only in GdmCl does the protein seem to be largely devoid of secondary structure, as shown by the lack of a strong CD band.

Since low pH and high temperature have similar effects on toxin conformation, they might be expected to have interacting effects on the triggering of the transition from a native to a nonnative state. Indeed, as shown in Fig. 5, lower pH shifts the thermal transition to lower temperatures. At very low pH the thermal transition is abolished. To obtain a more complete picture of the interaction of low pH and high temperature, the pH and thermal transition ranges were determined in a series of fluorescence intensity experiments in which either pH or temperature was varied. These data are summarized in Fig. 6, a state diagram that identifies both the conformational state at any given temperature–pH combination and the boundary between states (28–30). It confirms that low pH and high temperature interact such that as pH is decreased the thermal transition temperature progressively decreases and, above 30°C, as temperature is increased the transition pH increases. The narrow range of the transition suggests that the conformational change is highly cooperative all along the conformational boundary between "native" and "nonnative" states. This type of sensitivity to both low pH and high temperature is similar to that commonly observed for protein denaturation (see *Discussion*) (30). The state diagram also shows the much more gradual change between the high-temperature and low-pH states, indicating that a less cooperative or even noncooperative change is involved.

DISCUSSION

It has long been known that low pH and high temperature cause changes in diphtheria toxin conformation, and these

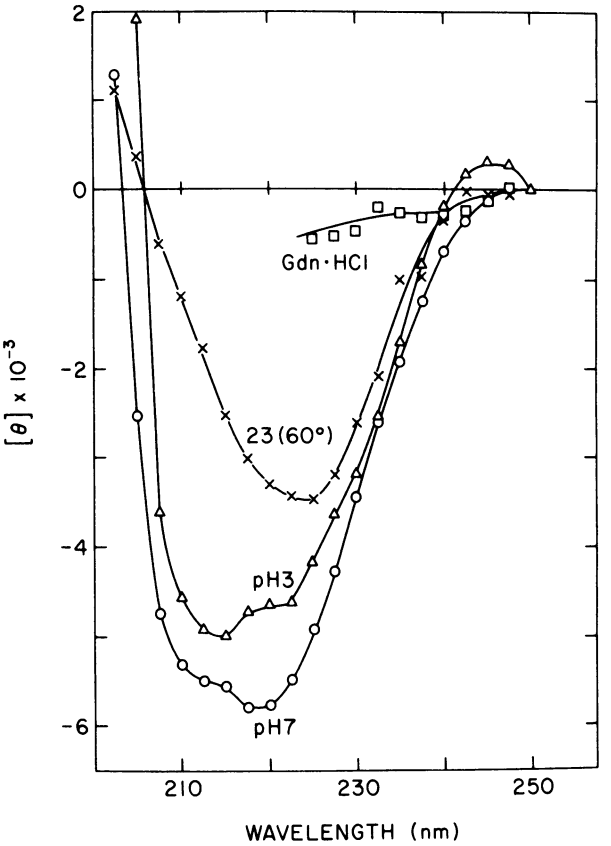

FIG. 4. CD of different toxin conformations. Units for [θ] are degrees·cm²/dmol. Samples contained protein at 25 μg/ml in 150 mM NaF/10 mM Tris·HCl, pH 7, unless otherwise noted. ○, Native toxin; △, toxin in 10 mM formate/150 mM NaCl, pH 3; ×, toxin incubated at 60°C, CD measured at 23°C; □, toxin in pH 7 buffer/150 mM NaCl/3 M GdmCl. The samples, except the last one, had 30% (vol/vol) glycerol to minimize any effects due to aggregation. We found that this concentration of glycerol did not significantly alter the thermal transition temperature.

changes were assumed to reflect denaturation (38). However, the exact nature of the conformational changes and their relationship to membrane penetration had not been studied. This report shows that the low-pH and high-temperature conformations are similar, as judged by their low fluorescence intensity, red-shifted emission λ_{max}, hydrophobicity, and acrylamide-detected tryptophan exposure. These changes involve at most only partial unfolding as judged by CD and tryptophan exposure experiments. There are also distinct differences between the conformations at low pH and high temperature, including the amount of the red shift, the degree of detergent interaction, and the degree of aggregation.

The conformation of the toxin in 3 M GdmCl is close to a random coil as judged from tryptophan properties and CD. Nevertheless, the toxin remains hydrophobic in this state. This means that hydrophobicity is not solely a result of tertiary structure interactions. This is consistent with toxin primary structure, which includes several long strings of hydrophobic residues (1–3).

The experiments in this report also show that low pH and high temperature interact such that they impart "additive" sensitivity towards disruption to the native, neutral-pH conformation. This implies there must be a region whose conformation can be disrupted by either of these extremes. A nondenaturing conformational change could exhibit such behavior. For example, in hemoglobin, destabilization of the oxygen-binding conformation occurs both when pH is de-

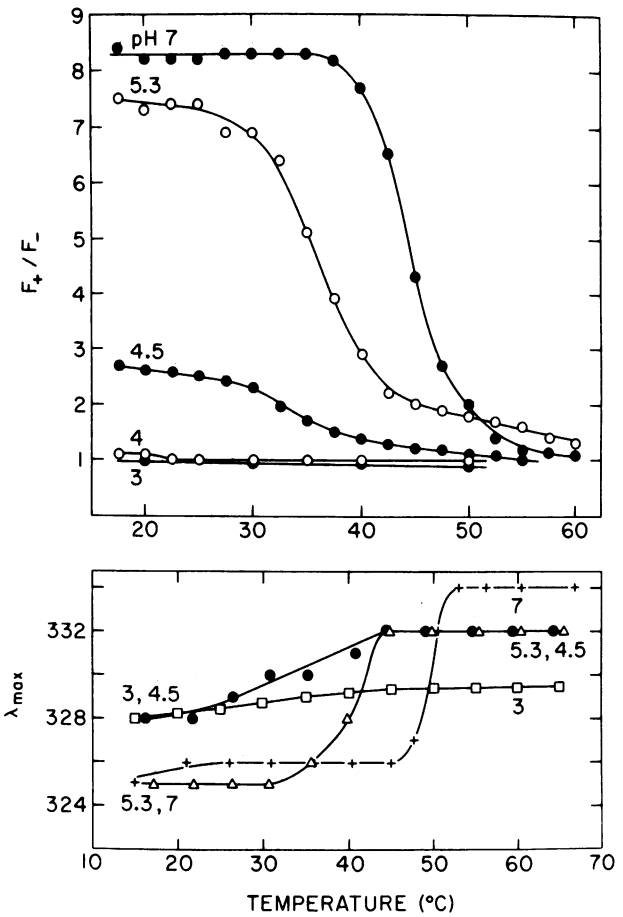

FIG. 5. Effect of pH upon the temperature dependence of toxin fluorescence. (*Upper*) Fluorescence intensity vs. temperature at various pH values. Fluorescence upon increasing temperature (F_+) was divided by fluorescence obtained during decreasing temperature scan (F_-) to cancel out ordinary temperature dependence of fluorescence (see text). (*Lower*) Dependence of λ_{max} upon temperature at various pH values. +, pH 7; △, pH 5.3; ●, pH 4.5; □, pH 3. For sample conditions see Fig. 1. The pH 7 sample contained Tris buffer in place of phosphate. At 50°C its pH is about 6.2.

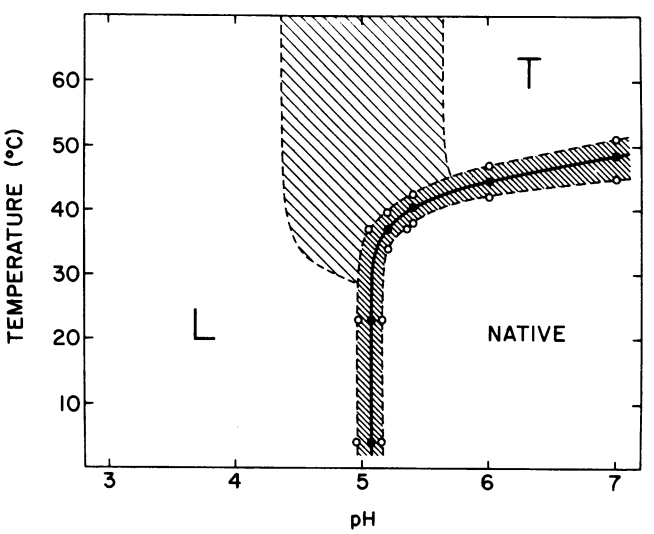

FIG. 6. Partial state diagram for diphtheria toxin monomers. The solid line represents the midpoint of the boundary between the native and nonnative states. L, low-pH conformation; T, high-temperature conformation. The middle half of each transition is shaded (i.e., between 25% and 75% complete). The data points, obtained from curves of fluorescence intensity and λ_{max} vs. temperature or pH, like those in Fig. 1 and 5, are shown for the midpoint of the transition (●), and for 25% or 75% completion of the transition (○).

creased (Bohr effect) and when temperature is increased (31). However, the changes undergone by the toxin are probably more drastic. Since the effect of high temperature upon the toxin is to destroy the interactions stabilizing the native conformation, as shown by the endothermic thermal transition, which is characteristic of denaturation (23), the low-pH conformational change must also involve the elimination of interactions stabilizing the native conformation, consistent with the similarity of toxin properties at low pH and high temperature. Therefore, we suggest that the low-pH changes in toxin conformation correspond to partial denaturation of a limited region, which functions as a step necessary for membrane penetration because it exposes a hydrophobic site. How could such a change expose the hydrophobic sites on the toxin? One obvious possibility is that a region covering the hydrophobic site could unfold. For example, it has been noted that hydrophobic sites are exposed when the C-terminal region of the toxin is removed, or when sodium dodecyl sulfate, which presumably causes some unfolding, is added to the toxin (32). However, there is as yet no evidence that this region undergoes a change at low pH. Alternately, a small linker region holding two domains together could unfold to expose sites at the interdomain interface, as has been suggested in the case of staphylococcal α-toxin (33). In future studies we hope to identify whether the changes at high temperature and at low pH involve part or all of either the A or B subunit, or both.

It may well be that other proteins that undergo similar conformational changes at low pH go through a similar process. There are several similarities between the pH behavior of influenza virus hemagglutinin and diphtheria toxin (20). Investigators working with influenza virus have hinted that for the viral hemagglutinin protein high temperature and low pH may have similar effects (34). On the other hand, they have suggested that the change in the hemagglutinin does not involve denaturation because of the lack of unfolding of secondary structure, as judged by CD. However, since the unfolding of a very small region would be hard to detect, the similarity between hemagglutinin and diphtheria toxin behavior may in fact be rather close. In fact, the behavior of the toxin and virus protein may be similar enough that the lysosomotropic drug amantadine, used prophylatically and therapeutically for influenza (perhaps because it increases pH in acidic organelles and prevents viral entry), should be considered for therapeutic use in diphtheria.

Since the physiological temperature for toxin action *in vivo* is 37°C, the state diagram suggests that a mixed conformation involving elements of the high-temperature and low-pH conformation will be triggered *in vivo* at the pH in acidic organelles [probably endosome, pH ≈ 5–5.5 (35–37)]. However, this can only be a tentative conclusion since other factors not yet fully explored, such as receptor- and lipid-binding effects, membrane potential, precise endosomal ionic conditions, aggregation, etc., may alter the state diagram. The role of these additional factors on toxin conformation requires further study. In any case, it is clear that care must be taken in comparison of measurements taken at different temperatures.

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