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FLUORESCENCE CHARACTERIZATION OF THE LOW pH-INDUCED CHANGE IN DIPHTHERIA TOXIN CONFORMATION: EFFECT OF SALT

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We have examined the effect of pH on diphtheria toxin conformation using intrinsic protein fluorescence and a new fluorescence quenching method. In aqueous solutions, fluorescence indicates toxin conformation undergoes a drastic change at low pH. This conformational change is closely associated with a switch from a hydrophilic conformation to a hydrophobic one, as judged by quenching-detected detergent binding. In the absence of NaCl these changes occur around pH 4-4.5. However, in 150 mM NaCl the conformational change occurs in the pH 5-5.5 range, close to the pH the toxin is expected to encounter in endosomes and lysosomes. Therefore, the conformational change observed at low pH is likely to be physiologically signficant.

It is now clear that in some cases there are strong similarities between virus and protein toxin entry into cells. Diphtheria toxin, Semliki Forest virus and influenza virus enter cells by attaching to receptors, undergoing endocytosis, and then penetrating the membrane of acidic vesicles, either endosomes or lysosomes (1-4). This latter step is accomplished partly by a low pH-induced switch from a hydrophilic conformation to a hydrophobic one. A previous study indicated that toxin hydrophobicity, inferred from Triton X-100 binding by normal toxin, becomes significant below the pH 4-4.5 range (4).

Our recent preliminary studies have shown that low pH alters toxin secondary and tertiary structure (5). In this report, we find that salt concentration influences the pH of the conformational switch. Without NaCl hydrophobicity is significant only below pH 4-4.5, in agreement with the previous results noted above. However, in 150 mM NaCl toxin becomes hydrophobic around pH 5, close to physiological values.

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MATERIALS AND METHODS

Crude diphtheria toxin was purchased from Connaught Laboratories (Ontario, Canada). Trypsin, N-α-p-tosyl-L-lysine-chloromethylkentone, and Brij 96 (an oleyl polyoxyethylene ether detergent) were purchased from Sigma Chemical. All other chemicals were reagent grade.

Purified toxin was isolated as described previously (6). The dimer toxin fraction with bound ApUp (a natural dinucleotide ligand (7)) was used for further experiments. Only a few percent of nicked toxin was present as judged by SDS gel electrophoresis. Protein concentration was calculated using a value of $\varepsilon_{280} = 55{,}300\ cm^{-1}M^{-1}$ for ApUp-bound toxin derived from published values for aromatic amino acids and ApU (8). (Lowry assay gave roughly 20% higher values).

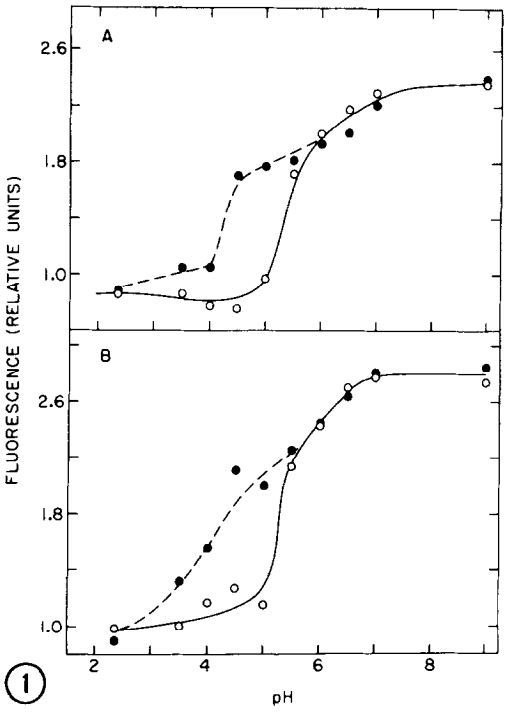
Toxin nicked between the A and B subunits was prepared by incubation of 10.8 mg intact toxin in 0.75 ml of 1.3 µg/ml trypsin /50 mM Tris-Cl pH 7.2/2mM $CaCl_2$. After 1h 5 µl of 1 mg/ml N-α-p-tosyl-L-lysine-chloromethylketone was added. Then toxin was reisolated by chromatography on a 2.5 cm x 95 cm column of Sephacryl S-200 using a buffer of 50 mM Tris-Cl pH 7.2/1 mM EDTA 0.02% (w/v) NaN_3. SDS gel electrophoresis showed that nicking was complete.

Dibrominated Brij 96 was synthesized by titration of an aqueous solution of Brij 96 with concentrated bromine water. The reaction was over in 5 seconds as monitored by loss of bromine color. Following excess bromine by A_{393} we found a sharp endpoint to the titration indicating that 70% of Brij 96 molecules contained double bonds in their hydrocarbon chains (the site of bromination). The Brij 96 used for further experiments was brominated to 80% of this maximal value to avoid residual free bromine.

Fluorescence was measured in 1 cm quartz cuvettes using a Spex 212 Fluorolog Spectrofluorimeter. Excitation and emission slits were used with nominal bandpass of 4.5 nm. Excitation was at 280 nm and emission was scanned from 300 to 360 nm. Background without toxin was subtracted from all reported values.

RESULTS AND DISCUSSION

Figure 1 shows the effect of pH on fluorescence of aqueous solutions of toxin. At low pH there is a marked drop in the toxin fluorescence intensity. In intact toxin the drop in fluorescence as pH is decreased shows a gradual 20-25% decrease first, followed by a rapid "transition" to a low fluorescence state. The transition occurs at pH 4-4.5 without NaCl and at pH 5-5.5 in 150 mM NaCl. It is accompanied by a red shift in λmax from 326 nm to about 333 nm below the transition pH. These results indicate that a conformational change takes place at low pH. In general, the behavior of nicked and intact toxin is similar. We find reproducibly that without NaCl the change in fluorescence at low pH is more gradual for the nicked toxin. However, it should be noted that in some of our toxin preparations fluorescence is constant above the transition pH. At present we do not understand the source of this variability.



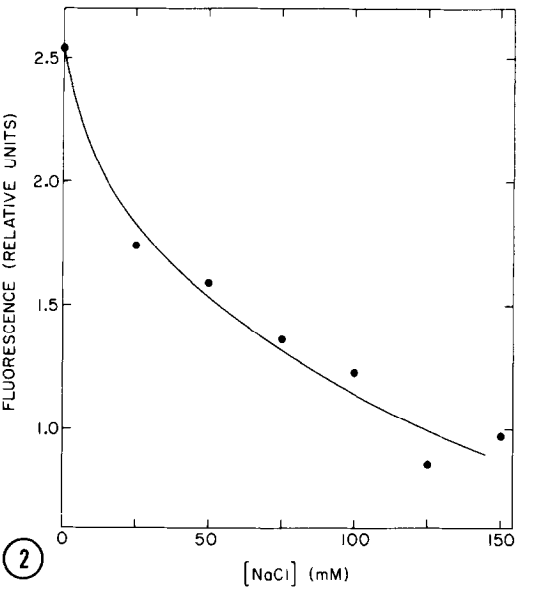


Figure 1: Effect of pH upon Fluorescence of Diphtheria Toxin. (A) Intact toxin (4.5 µg/ml). (B) Nicked toxin 5.1 µg/ml. Samples contained toxin in 10 mM buffer with (——) or without (---) 150 mM NaCl. Na phosphate buffer was used at pH 2.3 and 6 to7; Na formate buffer at pH 3 to 4.5. Na acetate buffer was used at pH 5 and 5.5; tris-Cl buffer was used at pH 9. Samples were incubated 1h before fluorescence at 330nm was measured.

Figure 2: Effect of NaCl upon Toxin Fluorescence at pH 5. Concentrated NaCl was added to a sample of 4.4 µg/ml intact toxin in 10 mM Na acetate pH 5. Fluorescence was read 5 min. after each addition of salt.

Figure 2 shows that at pH 5 the weakly fluorescent conformation is gradually stabilized as NaCl concentration is increased. This implies that the pH of the transition is sensitive to the exact amount of NaCl present.

Using brominated Brij 96 as a fluorescence quenching detergent the effect of pH upon detergent binding was examined. With this method detergent binding can be conveniently detected (5). Brij 96 was chosen because it is an easily brominated member of the mild, non-ionic polyoxyethylene class of detergents, which includes Triton X-100. Samples of toxin were incubated with brominated and unbrominated Brij 96 at each pH. Detergent binding was detected via quenching of tryptophan fluorescence, expressed as the ratio of fluorescence with brominated Brij 96 relative to that with unbrominated Brij 96. As shown in figure 3, detergent binding appears at pH 4-4.5 without NaCl

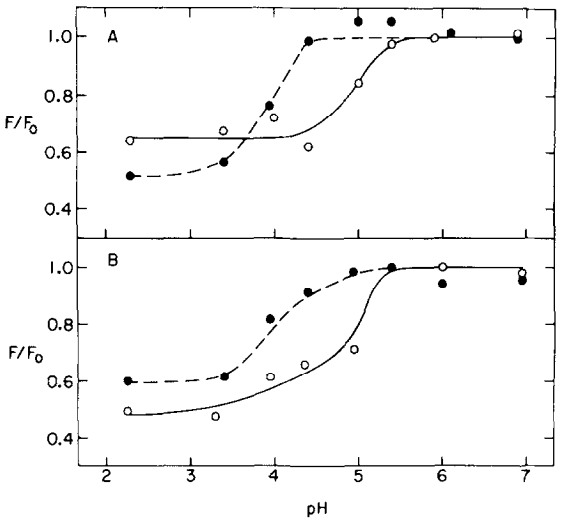


Figure 3: Effect of pH upon Quenching of Toxin Fluorescence by Brominated Detergent. (A) Intact Toxin (B) Nicked Toxin. Samples prepared as in figure 1 except that 0.1% (w/v) of either Brij 96 or dibrominated Brij 96 was present. Symbols same as Figure 1. Ordinate is the ratio of fluorescence in the presence of dibrominated Brij 96 (F) to that in the presence of Brij 96 (F_o).

and around pH5 in 150mM NaCl. Therefore, the pH at which detergent binding appears is close to or slightly lower than the pH of the fluorescence transition. It is probable that the transition in toxin conformation is associated with either the formation or exposure of a hydrophobic site on the protein.

The pH of 4-4.5, at which Brij binding appears without NaCl, is very close to that reported previously (4) for Triton X-100 binding under similar ionic conditions, using a much more laborious sucrose gradient method. The value of pH 5 found in 150 mM NaCl is closer to physiological pH. This range of pH is about what the toxin should encounter in lysosomes or endosomes. Therefore, we now can conclude that it is likely that the acid induced toxin hydrophobicity is physiologically significant. Because the quenching bromine atoms are attached to the detergent's hydrocarbon chain another conclusion of these experiments is that a considerable fraction of the fluorescent tryptophan residues are exposed to the hydrophobic region of the micelles. At low pH, the stronger quenching of nicked toxin relative to intact toxin in 150 mM NaCl could either involve a difference in tryptophan exposure or the

amount of bound detergent. In either case it is likely that tryptophan residues come into contact with membrane lipids *in vivo*.

Even with these results, the precise story of pH control of toxin conformation is not complete. Several additional variables may affect the pH of the hydrophilic to hydrophobic transition. For example, various ions, lipids, a pH gradient, membrane potential and other environmental factors may affect the transition. Furthermore the toxin can exist in many other forms including monomer, separated subunits, free of ApUp or with reduced disulfide. Any of these forms could differ in hydrophobicity. For example, the mutant CRM 45 toxin, missing amino acids from the C-terminal of its B fragment, binds Triton X-100 at near neutral pH (9). Furthermore, receptor-binding to toxin may also affect hydrophobicity. Clearly, more work is needed.

Fluorescence quenching by spin-labeled (10,11) and brominated (12,13) lipids has recently been introduced as a powerful tool for elucidation of several aspects of lipid-protein interaction (14). This report shows that brominated detergents will also be a powerful tool for understanding membrane protein behavior.

ACKNOWLEDGEMENTS

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