Oliver, D., & Jagendorf, A. (1976) *J. Biol. Chem. 251*, 7168-7175.
Penefsky, H. S. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 111-138.
Schlimme, E., de Groot, E. J., Schott, E., Strottman, H., & Edelmann, K. (1979) *FEBS Lett. 106*, 251-256.
Schmid, R., Jagendorf, A. R., & Hulkower, S. (1977) *Biochim. Biophys. Acta 462*, 177-186.
Sharp, R. R., & Yocum, C. F. (1980) *Biochim. Biophys. Acta 592*, 185-195.
Sharp, R. R., & Frasch, W. D. (1985) *Biochemistry* (preceding paper in this issue).
Shoshan, V., & Selman, B. R. (1980) *J. Biol. Chem. 255*, 384-389.
Solomon, I., & Bloembergen, N. (1956) *J. Chem. Phys. 25*, 261.
Sugayama, Y., & Mukohata, Y. (1978) *FEBS Lett. 85*, 211-214.
Sugayama, Y., & Mukohata, Y. (1979) *FEBS Lett. 98*, 276-280.
Vallejos, R. (1981) in *Energy Coupling in Photosynthesis* (Selman & Selman, Eds.) Elsevier/North-Holland, Amsterdam.
Vallejos, R. H., Viale, A. M., & Andreo, C. S. (1977) *FEBS Lett. 84*, 304-308.
Webb, M. R., Grubmeyer, C., Penefsky, H. S., & Trentham, D. R. (1980) *J. Biol. Chem. 255*, 11637-11639.

Effect of pH on the Conformation of Diphtheria Toxin and Its Implications for Membrane Penetration†

Michael G. Blewitt, Laura A. Chung, and Erwin London*
Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794-5215
Received October 25, 1984

ABSTRACT: The pH-triggered change in diphtheria toxin conformation and the physical properties of the toxin above and below the transition pH have been examined. Exposure to low pH (≤5 at 23 °C, ≤5.3 at 37 °C) triggers a rapid ($t_{1/2}$ < 30 s) change in toxin conformation; the transition occurs over a narrow pH range (0.2 unit). Below the transition pH, buried tryptophans become exposed, and the toxin becomes hydrophobic, binding very tightly to detergent. Aggregation is observed at low pH, probably due to this extreme hydrophobicity. Circular dichroism and fluorescence properties show that the low-pH conformation is not extensively unfolded. Therefore, the toxin "opens" at low pH without becoming a random coil. The conformation change is partly irreversible, and the degree of irreversibility parallels the degree of aggregation. Reduction of the disulfide bonds does not increase hydrophobicity at neutral pH. Furthermore, none of the structural variants of toxin (monomer or dimer, bound to ApUp or free, and nicked between subunits or intact) are hydrophobic at neutral pH or differ in transition pH markedly. Therefore, these factors do not mimic the effect of low pH. These observations are consistent with a functional role for the pH-triggered changes during penetration of the membranes of acidic organelles. The toxin may have adapted a conformational change similar to partial denaturation for a critical role in function. The possible nature of the pH-sensitive interactions and the effects of aggregation are discussed briefly.

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 inactivates elongation factor 2 by ADP-ribosylation, and subunit B which binds to a receptor molecule and also is required to translocate subunit A into the cytoplasm. The structure of the toxin at neutral pH and its enzymatic function have been extensively studied (Collier, 1983; Pappenheimer, 1977; Uchida, 1983). The sequence of each subunit is now known (Greenfield et al., 1983; Kaczorek et al., 1983; Ratti et al., 1983). Several studies indicate that the toxin enters cells by receptor-mediated endocytosis, followed by penetration through the membrane of an acidic organelle (Draper & Simon, 1980; Sandvig & Olsnes, 1980, 1981). Indirect evidence suggests that acidic endosomes are the most likely site of membrane penetration (Marnell et al., 1984). The importance of low pH in membrane penetration by toxin is demonstrated both by the ability of lysosomotropic amines, which increase pH in acidic organelles, to inhibit toxicity and from the ability of low-pH incubation of cells with surface-bound toxin to overcome this block (Sandvig & Olsnes, 1980, 1981). Furthermore, such a low-pH incubation with surface-bound toxin can restore toxicity when endocytosis is blocked at low temperatures (Draper & Simon, 1980). In model systems, low-pH induction of toxin hydrophobicity (Blewitt et al., 1984; Sandvig & Olsnes, 1981) and low-pH-induced pore formation in membranes (Donovan et al., 1981; Kagan et al., 1981; Misler, 1983, 1984) have been observed. However, these studies have not yet explained the exact mechanisms of membrane penetration and of subunit A translocation.

In previous preliminary studies, we have noted a distinct change in toxin conformation at low pH (Blewitt et al., 1984; London et al., 1984). In this report, the conformational change at the pH transition has been further characterized, as well

† This work was supported by National Institutes of Health Grant GM31986 and by a University Award and a Biomedical Research Grant from the State University of New York to E.L.

0006-2960/85/0424-5458$01.50/0 © 1985 American Chemical Society

as the effect of varying environmental conditions and toxin structure upon the pH dependence of conformation.

Experimental Procedures

Materials. Diphtheria toxin was purchased from Connaught Laboratories (Ontario, Canda). Purification of the toxin in various forms has been previously described (Blewitt et al., 1984; Collier & Kandel, 1971; McKeever & Sarma, 1982). Toxin was stored at 4 °C. Its behavior was not dependent on storage time. However, the degree of nicking of nucleotide-free toxin did increase. Brij 96 and polidocanol [polyoxyethylene(9) lauryl ether; $C_{12}E_9$] were purchased from Sigma. Dithiothreitol (DTT)[1] ("high purity") was purchased from Calbiochem-Behring. Acrylamide ("electrophoresis grade") was purchased from Bio-Rad. Brominated Brij 96 was synthesized as previously described (Blewitt et al., 1984) except that stock solutions of Brij 96 and brominated Brij 96 were neutralized with NaOH before use. Brij 96 and brominated Brij 96 solutions were stored at room temperature with 0.02% (w/v) NaN_3.

Protein Determination. Protein concentration was estimated from the absorbance at 280 nm by using calculated values of ϵ_{280} = 55 000 M^{-1} cm^{-1} (1 OD_{280} = 1.06 mg/mL) for bound toxin and ϵ_{280} = 50 000 M^{-1} cm^{-1} for free toxin. These values were calculated from the published extinction coefficients of the aromatic amino acids, cystine, and ApU (Fasman, 1976). They are in good agreement with values estimated by other methods (Blewitt et al., 1984; Proia et al., 1981). An A_{260}/A_{280} ratio of 0.6 was obtained for free toxin and 0.9 for nucleotide-bound toxin, in agreement with previously reported values (Proia et al., 1981).

Fluorescence Spectroscopy. Fluorescence was measured by using a Spex 212 Fluorolog spectrofluorometer. Measurements were made on 2–2.5-mL samples in 1-cm path-length quartz cuvettes. Background intensity was subtracted from the reported values. Excitation slits of either 2.3- or 4.5-cm nominal band-pass and emission slits of 4.5-nm nominal band-pass were used. Intensity measurements at fixed wavelength were taken at 280-nm excitation and 330-nm emission unless otherwise noted. Excitation and emission maxima were determined from corrected spectra. Inner filter effects were negligible in all samples except those containing DTT and acrylamide.[2] Reported values are corrected for inner filtering (Leese & Wehry, 1978). Fluorescence lifetimes were measured with an Ortec 290 instrument. The light source was a gated N_2 flash lamp (with 1 atm N_2) (Photoresearch Associates, London, Ontario). Samples were irradiated at 295 nm, and emission at 320 nm was measured by using a cutoff filter (Schott WG 320, 3 mm). Samples contained 0.1 mg/mL intact bound dimer toxin in 148 mM NaCl and either 10 mM formate at pH 3.2 or 10 mM acetate at pH 4.4 or 10 mM NaP_i at pH 7. Background samples without protein had only negligible "emission" (2–5% of samples). Approximate lifetimes were determined by deconvolution.

HPLC and Native Gel Electrophoresis. A Beckman isocratic high-performance liquid chromatograph, equipped with a 7.5 × 300 mm Sepherogel-TSK 3000 SW column, was used for gel filtration measurements. Samples containing 25 μg of toxin were eluted at 0.5 mL/min in 75 mM NaP_i, pH 7. Approximate elution volumes were 9.5 mL for dimers and 10.5 mL for monomers, which was consistent with the values expected by using a calibration curve of protein standards. At pH 4.2, the elution buffer was 10 mM sodium acetate with 150 mM NaH_2PO_4, and at pH 3, 165 mM NaP_i was used.

Native gel electrophoresis was also used to evaluate toxin self-association. A modification of the procedure of O'Farrell was used (O'Farrell, 1975). A 10.1% (w/v) acrylamide gel [containing 0.13% (w/v) bis(acrylamide)] in 0.375 M Tris-HCl, pH 8.7, was prepared with a 5.1% (w/v) acrylamide stacking gel [containing 0.13% (w/v) bis(acrylamide)] in 62.5 mM Tris-HCl, pH 6.8. SDS and mercaptoethanol were omitted from all solutions. Toxin (10 μg in 20–40 μL) was incubated 1 h in 140–150 mM NaCl at pH 3, 4.2, or 7 and neutralized prior to electrophoresis.

Circular Dichroism. CD spectra were measured by using a Cary 60 instrument with a 6001 CD attachment. Quartz cuvettes with a 1-cm path length were used. The instrument was calibrated at 290 nm with (+)-camphorsulfonic acid (Aldrich Chemical Co.) and dried under high vacuum, assuming $[\theta]_{290}$ = +7.8 × 10^3 deg·cm^2/dmol (Tuzimura et al., 1977). After calibration, a value of $[\theta]_{220}$ = −10.3 × 10^3 deg·cm^2/dmol was obtained with (−)-pantolactone (Tuzimura et al., 1977). The apparent ellipticity of control samples lacking toxin was subtracted from all reported values.

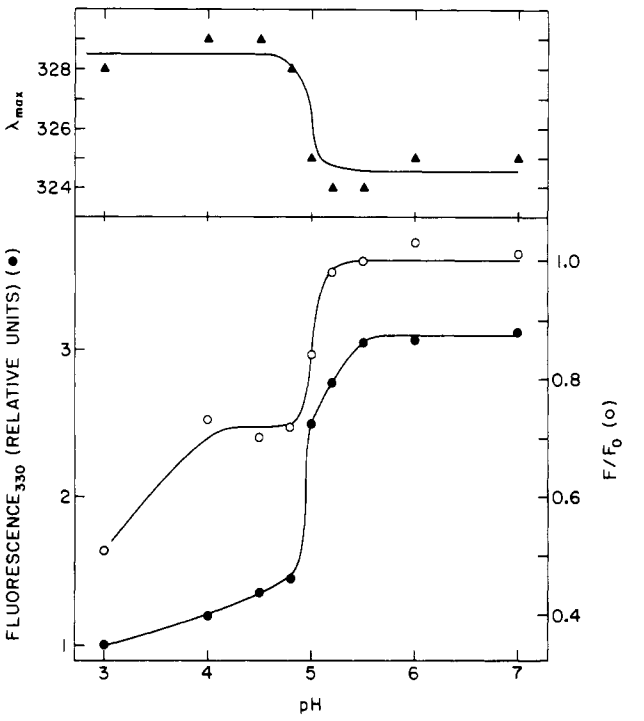

FIGURE 1: Effect of pH on the fluorescence of diphtheria toxin. (●) Fluorescence intensity; (○) quenching-detected Brij 96 binding; (▲) fluorescence emission λ_{max}. Samples of 5 μg/mL free dimer toxin in 150 mM NaCl and 10 mM buffer were incubated at ambient temperature 1 h before fluorescence was measured. Buffers used were formate at pH 4 and below, acetate from pH 4 to pH 5.5, and phosphate at pH >5.5. Samples for detergent binding studies contained 140 μM Brij 96 (0.01% w/v) or brominated Brij 96. F/F_0 is the ratio of fluorescence intensity in the presence of brominated Brij 96 to that in ordinary Brij 96 (see text for details).

Results

Effect of pH upon Toxin Fluorescence and Detergent Binding. Figure 1 shows the effect of pH upon the fluorescence of free (i.e., without bound ApUp) toxin dimers. Several changes can be identified. The intensity of fluorescence be-

[1] Abbreviations: DTT, dithiothreitol; P_i, inorganic orthophosphate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; CD, circular dichroism.

[2] Samples containing DTT had a considerable absorbance (1 cm) at 280 nm (0.05–0.15) arising from oxidized DTT formed during incubation.

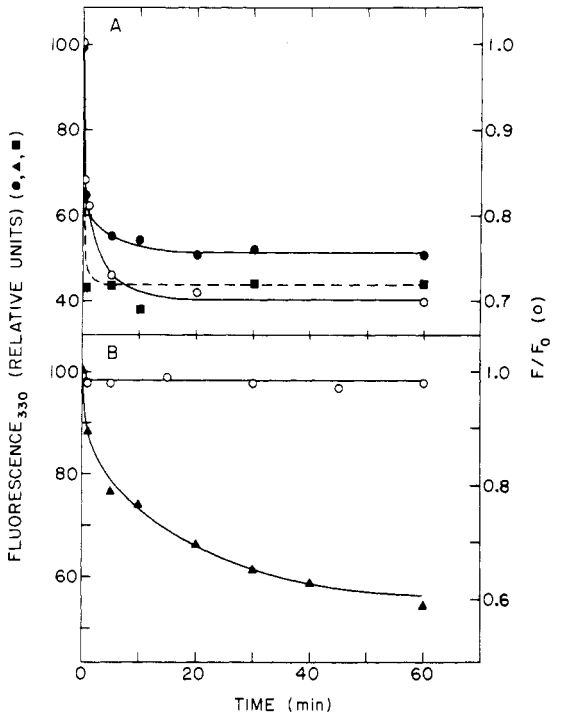

FIGURE 2: Kinetics of changes in toxin fluorescence. (A) Change induced by low pH: (●) fluorescence intensity at pH 4.2; (■) fluorescence intensity at pH 3; (○) quenching-detected detergent binding at pH 4.2 Samples at pH 7 were introduced to low pH at time zero. Other conditions as in Figure 1. (B) Changes upon addition of DTT at pH 7 to nicked free monomers (final concentration 50 mM): (▲) fluorescence intensity; (○) quenching-detected detergent binding. Other conditions as in Figure 1.

comes weaker at lower pH, with a sharp transition in fluorescence intensity near pH 5. The width of this transition is about 0.2 pH unit. A shift in the emission λ_{max} from about 325 to 329 nm also is observed below pH 5. The shift occurs at the transition pH.

Evaluation of the effect of pH upon toxin hydrophobicity is important for an understanding of toxin behavior. Binding of nonionic detergents can be used to detect protein hydrophobicity (Helenius & Simons, 1972). We used our recently developed method for fluorescence quenching detection of detergent binding (Blewitt et al., 1984) to examine the binding of Brij 96 to diphtheria toxin. In this technique, detergent binding is detected as a reduction of the fluorescence intensity (F) in brominated Brij 96 relative to the fluorescence intensity (F_0) in ordinary Brij 96 (i.e., by the degree of quenching). In figure 1, an F/F_0 value of close to 1 is observed above pH 5, indicating lack of toxin binding to detergent. Below pH 5, a drop in F/F_0, indicative of detergent binding, is observed. The fluorescence and hydrophobicity of free toxin are very similar to what we observed previously for bound toxin (Blewitt et al., 1984). The small difference in λ_{max} values from those we reported previously is largely due to the fact that the present data come from corrected fluorescence spectra (see Experimental Procedures).

The kinetics of the changes induced by low pH are shown in Figure 2A. The transition from the high-pH to the low-pH conformation is rapid, with the change more than 50% complete in less than 30 s. The kinetics are faster at pH 3 than at pH 4.2. Hydrophobicity appears at virtually the same rate as the fluorescence decrease, indicating that these two changes are closely linked.

The strength of Brij 96 binding to toxin at pH 4.2 is illustrated in Figure 3. About 50% of maximal quenching occurs in 0.0003% (w/v) (4 μM) Brij 96. Indeed, association

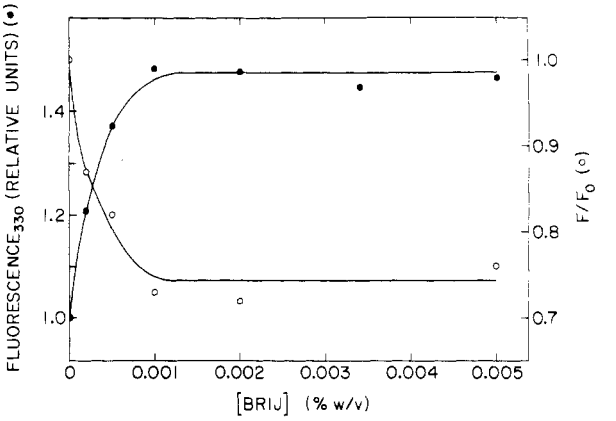

FIGURE 3: Binding of detergent to toxin at pH 4.2. (●) Fluorescence intensity in the presence of various amounts of Brij 96; (○) quenching-detected detergent binding. Samples contained Brij 96 or equimolar brominated Brij 96. Each point represents separately prepared samples. Other conditions as in Figure 1.

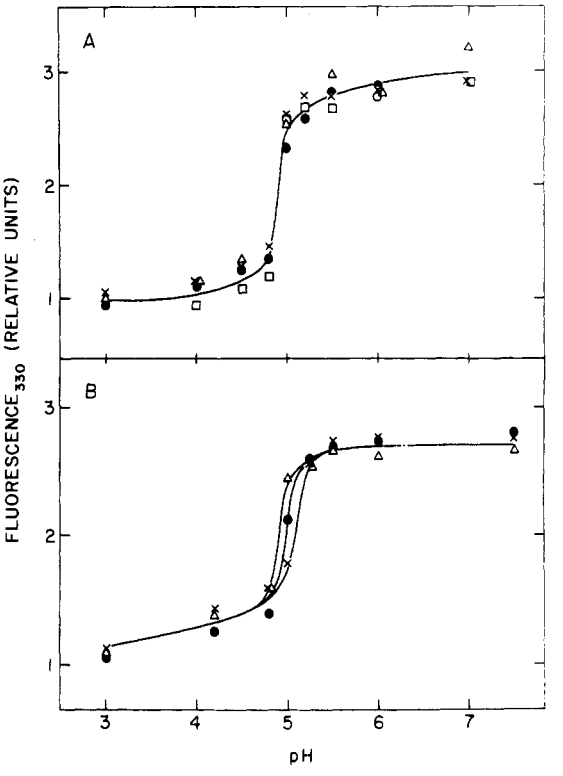

FIGURE 4: Effect of salts and form of toxin upon pH dependence of fluorescence. (A) Fluorescence in various salts: (●) 150 mM NaCl and 10 mM buffer; (×) 150 mM KCl and 10 mM buffer; (□) 100 mM NaP_i (and 10 mM buffer from pH 4 to 5.5); (△) 150 mM NaF and 10 mM buffer (except 56.5 mM HF as buffer at pH 4.5 and 225 mM HF at pH 4). Other conditions as in Figure 1. (B) Fluorescence of different forms of toxin: (●) free dimers; (△) intact, bound dimers; (×) nicked free monomers. Other conditions as in Figure 1.

with such a low concentration of detergent indicates very tight binding to detergent,[3] and therefore the potential for tight binding to a membrane bilayer. Figure 3 also shows that protein fluorescence intensity increases upon addition of ordinary Brij 96 to the aqueous solutions of toxin. The fluorescence enhancement exactly mirrors the fluorescence

[3] This experiment may even underestimate binding strength since the toxin presumably binds primarily to micelles of Brij 96 and the (unusually low) critical micelle concentration of Brij 96 is close to 0.00025% (w/v) (A. Chattopadhyay and E. London, unpublished observations).

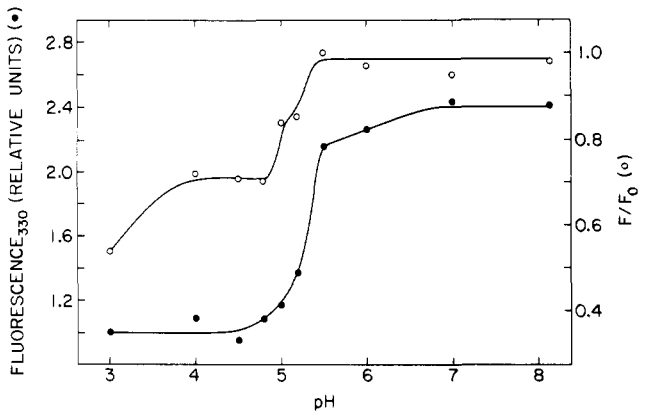

FIGURE 5: Fluorescence of toxin at 37 °C. Free dimer toxin was incubated at 37 °C for 90 min, and then fluorescence was measured in a thermostated cuvette. pH was measured at 37 °C. Brij 96 or the brominated Brij 96 concentration was 700 μM in samples containing detergent. Similar results were observed in 140 μM detergent. Other conditions as in Figure 1.

quenching by brominated Brij 96 which indicates that both changes measure binding to detergent.

Influence of Environment and Toxin Structure upon the pH Transition. In previous studies, we identified NaCl concentration as a major control of the transition pH, which occurs near pH 4 in low salt and at pH 5 in 150 mM NaCl (Blewitt et al., 1984). Figure 4A shows that the effect of salt is independent of the type of ions present for a number of monovalent salts, at similar ionic strength. Also, when in addition to 150 mM NaCl either 10 mM $MgCl_2$ or 10 mM $CaCl_2$ was added, the fluorescence behavior vs. pH was not affected (data not shown). These results imply that in these cases ionic strength, not the type of ion, is important.[4]

The behavior of various forms of the toxin is compared in Figure 4B. There seems to be only a small difference in the pH transition among the forms tested, with the transition of nicked, free monomers perhaps 0.2 pH unit above that of intact, bound dimers. The observation that both free monomers and bound dimers show a very similar high- to low-pH transition proves that changes in either ligand binding or the monomer/dimer equilibrium are not primarily responsible for the observed changes in fluorescence intensity. The pH dependence of detergent binding by monomers and dimers is also very similar (data not shown). Since the behavior of different forms is so similar, a preparation of free dimers containing some nicked toxin was used in most of our studies for reasons of convenience.

Further experiments demonstrate that higher temperature can also alter the transition pH. At 37 °C, the pH transition is shifted upward about 0.3 pH unit to pH 5.3 (Figure 5). This may have important implications concerning the structure of the low-pH conformation (see Discussion).

Another factor that has been examined is the state of the disulfide bonds. The disulfide bonds of the toxin play an important role in its structure. Toxin disulfides can be reduced by incubation in 50 mM DTT (Collier & Kandel, 1971; Gill & Dinius, 1971). Figure 6 shows incubation of toxin in DTT alters the effects of pH. The samples were preincubated in DTT at pH 7 and then transferred to low pH. The reason for this preincubation is to avoid the negligible rate of reduction at low pH.[5] At pH 7, the fluorescence intensity decreases

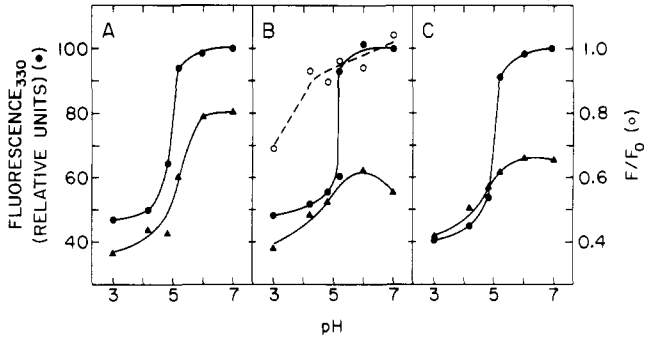

FIGURE 6: Effect of DTT on toxin fluorescence. Samples contained toxin incubated in the (●) absence or (▲) presence of 50 mM DTT at pH 7 for 45 min and then diluted into buffer containing no DTT (●) or 50 mM DTT (▲) at the appropriate pH and incubated for 1 h. (○) Quenching-detected detergent binding in the presence of 50 mM DTT. (A) Intact bound dimers. (B) Nicked free monomers. (C) Free dimers. Other conditions as in Figure 1.

in DTT, leveling off in about 1 h (Figure 2A). In general, there is a much smaller decrease in fluorescence at low pH than at pH 7, as shown in Figure 6. Interestingly, the effect of DTT depends on the form of toxin used. Intact bound dimers show a weaker but still prominent pH transition while free dimers and nicked free monomers do not show a transition but a gradual decrease of fluorescence at low pH.

Incubation in DTT also affects hydrophobicity. In these experiments, detergent was added and sample pH changed subsequent to reduction. As shown in Figure 6, nicked free monomers only show slight quenching above pH 3, with no hint of a transition at pH 5. Aggregation and detergent binding may compete (see Discussion). Therefore, to see if irreversible aggregation during preincubation in DTT at pH 7 was responsible for the lack of subsequent detergent binding, toxin was incubated simultaneously in DTT and detergent at pH 7. As shown in Figure 2A, no quenching is observed at any time examined.

Reversibility of the Effects of Low pH. According to most models of toxin action, the toxin travels from a neutral pH environment to a low-pH compartment and then becomes at least partially exposed to near-neutral cytoplasmic pH after membrane penetration. For this reason, the degree of reversibility of the effects of low pH is of interest. Figure 7 shows the effects of reversing the pH to 7 upon fluorescence intensity and detergent binding. The drop in fluorescence intensity is almost completely irreversible, except at pH 3 where a reproducible small increase is observed upon reversing the pH. A similar pattern is observed for quenching-monitored detergent binding. However, reversibility of detergent binding at pH 3 is nearly complete. These observations are in agreement with the studies of Sandvig & Olsnes (1981) which demonstrated that temporary incubation of toxin at low pH has largely irreversible effects for cytotoxicity activity at pH 4–4.5 but has mostly reversible effects when low-pH incubation is done below pH 3.5.

Measurements of pH-Induced Conformational Changes Using Aqueous Quenching and Circular Dichroism. To confirm and further characterize the effects of pH upon toxin, the conformation was evaluated with an aqueous quencher of fluorescence and by measurement of circular dichroism. Aqueous fluorescence quenchers, such as acrylamide, measure exposure of Trp residues to the aqueous environment (Eftink

[4] It should be noted that we have not examined our toxin preparations for endogenous bound ions. EDTA is present during isolation of the toxin (McKeever & Sarma, 1982).

[5] Disulfide bond exchange and reduction are catalyzed by the anionic (S^{-1}) form of thiols and thus are strongly inhibited by low pH (Torchinsky, 1981).

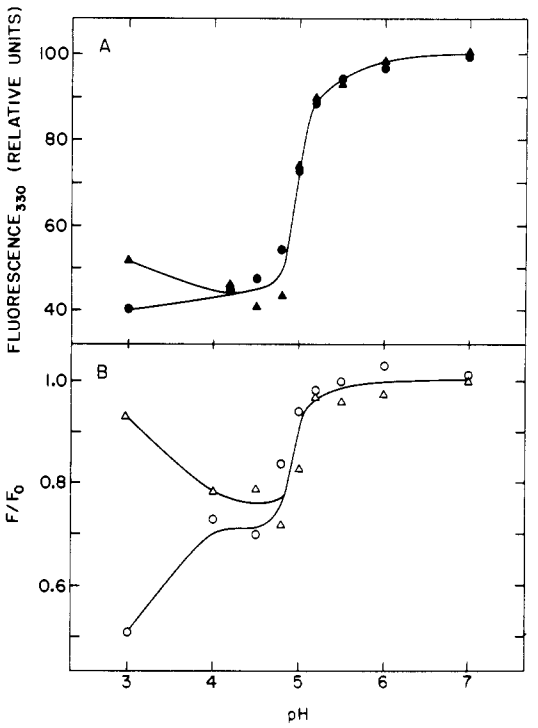

FIGURE 7: Reversibility of the effect of pH upon fluorescence. Samples of 2 mL containing free dimer toxin were incubated at various pHs for 60 min, and then the pH was reversed to pH 7 by addition of an aliquot of 0.4 M Na_2HPO_4. Fluorescence was then read after a further 15-min incubation. (A) Fluorescence intensity (●) before and (▲) after pH reversal. (B) Quenching-detected detergent binding (○) before and (△) after pH reversal. Fluorescence values shown were corrected for dilution. Other conditions as in Figure 1.

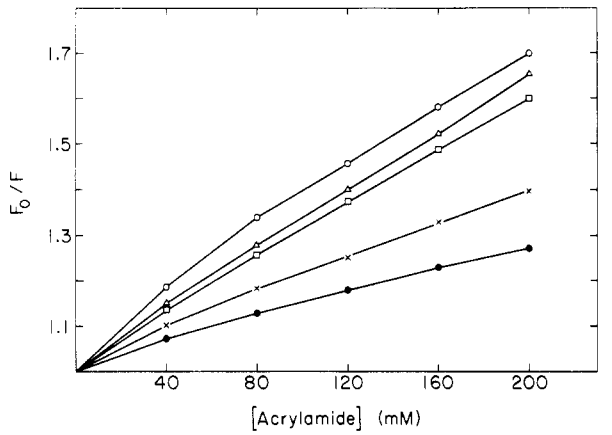

FIGURE 8: Quenching of toxin fluorescence by acrylamide. Samples incubated as described in Figures 1 and 7. Free dimer toxin at (●) pH 7, (○) pH 3, (△) pH 4.2, (□) pH 7 after incubation at pH 4.2 and (×) pH 7 after incubation at pH 3. Each sample was "titrated" with acrylamide, and fluorescence intensity at λ_{ex} = 295 nm and λ_{em} = 340 nm was measured after each addition.

& Ghiron, 1976, 1981). The higher the slope of an F_0/F vs. [quencher] curve, the greater the degree of exposure, provided the fluorescence lifetime does not increase. Acrylamide quenching of free dimer toxin is shown in Figure 8. Trp residues are much more exposed at low pH, with nearly equal exposure at pH 3 and 4.2. Similar results were obtained with toxin monomers and bound toxin (data not shown). The quenching is reversed substantially upon reversing the pH from 3 to 7 but not when reversing the pH from 4.2 to 7, in agreement with the properties of the toxin noted above. The fluorescence lifetime of toxin is roughly 4 ns at pH 7 and between 3 and 4 ns below pH 5. Therefore, the increase in

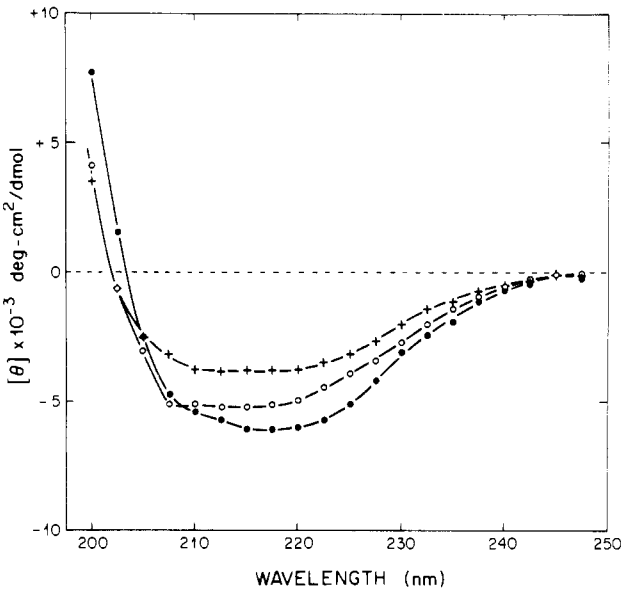

FIGURE 9: Circular dichroism of toxin: (●) pH 7; (○) pH 3; (+) pH 4.4. Samples contained 25 μg/mL free dimer toxin at 21–23 °C. Spectra are the average of two samples. Other conditions as in Figure 1.

quenching at low pH is not due to an increase in lifetime.

The circular dichroism of the toxin in the region 200–250 nm is shown in Figure 9. At neutral pH, the shape of the CD spectrum indicates that the toxin is rich in β-sheets. There appears to be relatively little α-helix [5–20% maximum estimated by the methods of Greenfield & Fasman (1969) or by the data of Chen et al. (1974)]. However, the spectral shape is equally consistent with an α/β-type protein as well as a β-sheet-rich conformation (Manavalan & Johnson, 1983). At low pH, the ellipticity of the toxin changes slightly, with ellipticity less negative around 220 nm and more negative below 210 nm. These changes suggest a slight increase in random-coil at low pH. In view of the aggregation of toxin at low pH (see below), the possibility of differential scattering and absorbance flattening artifacts (Bustamante et al., 1983; Simons, 1981) must be considered. Since such effects result in less negative ellipticity, especially at lower wavelengths (Simons, 1981), they cannot explain the changes below 210 nm. However, the changes in intensity between 210 and 240 nm may well be due to aggregation (see below). Despite this artifact, it is clear the toxin does not become a random-coil below pH 5.

Effect of pH upon Toxin Self-Association. Toxin can be either monomeric or dimeric at neutral pH (Collier, 1982; Pappenheimer, 1977; Uchida, 1983). The effect of pH upon the degree of self-association of dimer toxin was determined by HPLC gel filtration. At pH 3, most toxin eluted faster (i.e., of larger size) than dimer, although a dimer peak was also present. The recovery of protein was only 10–20%. When the sample pH was reversed to 7, the high molecular weight peaks observed at pH 3 were replaced by a dominant monomer peak indicating some disaggregation. However, the percent recovery did not increase. The low recovery suggests the presence of larger aggregates that fail to elute and which do not dissociate upon reversing the pH to 7. At pH 4.2, aggregation was more severe, as judged from a recovery of only 1%. This recovery was not increased by reversing the pH.

Native gel electrophoresis gave similar results. Gel samples consisted of dimer toxin incubated at pH 3, 4.2, or 7 and then neutralized before electrophoresis. Incubation at pH 3 or 4.2 resulted in aggregation of toxin, as shown by strong staining

at the top of the gel. However, monomer and dimer bands were also observed in the samples incubated at pH 3 and 4.2. Monomer and dimer bands were weakest at pH 4.2, indicating aggregation was most severe at this pH. Interestingly, after incubation at low pH, the monomer band stained more strongly than the dimer band. Therefore, low-pH incubation seems to destabilize the dimer in the disaggregated population.

Discussion

One question partly answered by this study is what conformational changes occur in diphtheria toxin at low pH. As noted by previous investigators, the toxin appears to have a buried hydrophobic site (Boquet et al., 1976) exposed by low pH (Sandvig & Olsnes, 1981). Our results confirm this and also show that exposure of buried Trp-containing sites occurs at low pH. This is shown by the red shift of tryptophan fluorescence emission and the increase in acrylamide quenching of tryptophan at low pH. On the other hand, the fluorescence emission λ_{max} and the slope of the acrylamide quenching curve indicate incomplete exposure of tryptophans at low pH (Eftink & Ghiron, 1976). Circular dichroism shows at most only a small change in secondary structure from an apparent β-sheet-rich conformation to one with a small amount of additional unordered conformation. Therefore, below pH 5, a switch to a more "open" conformation, in which unfolding is not extensive, occurs. Combining this conclusion with the observation that higher temperature destabilizes the neutral pH conformation, shifting the transition to a higher pH, and with preliminary studies that reveal some similarities between the low-pH and thermally denatured conformation[6] leads us to believe that at low pH the toxin undergoes a change that is similar to a partial denaturation but which has been adapted for a functional role. Our present studies are directed toward exploring this idea.

Factors other than low pH and high temperature might conceivably give rise to a conformational change similar to that induced by low pH or might regulate the effect of pH. The observations in this report suggest that the various variants in toxin structure (i.e., monomer or dimer, free or bound to ApUp, niked or intact) do not have such a role. In addition, reduction of the disulfide bonds does not seem to give rise to a hydrophobic state. In fact, since reduction is unlikely in the oxidizing external medium outside the cell and is inhibited in a low-pH environment[5], it would seem likely that reduction occurs subsequent to membrane penetration.

Therefore, the experiments in this report are consistent with the proposal that low pH is the main factor triggering membrane penetration. Three physical properties of the toxin that support this statement the following: (1) the transition occurs at pH 5.3 at 37 °C, which is similar to the pH found in endosomes (Geisow & Evans, 1984; Maxfield, 1982) and lysosomes (Geisow, 1984); (2) detergent binds very tightly to the protein at low pH, and tight binding to the hydrophobic portion of the bilayer would be expected for efficient penetration of membranes by the toxin (not to be confused with tight *extrinsic* binding of the toxin to the membrane via a receptor); (3) the kinetics of the low-pH-induced conformational changes easily meet the condition that they must be fast enough to occur within a few minutes, the time scale of cytotoxicity appearance in cell culture (Marnell et al., 1984).

Another line of evidence consistent with a physiological role for the low-pH-induced transition comes from the parallels between the behavior of toxin and that of the fusion proteins of viruses that penetrate acidic organelles. The pH needed for influenza virus fusion is very similar to the pH of the toxin transition (Huang et al., 1981; Maeda & Ohnishi, 1980; White et al., 1981). Furthermore, fusion occurs rapidly upon exposure to low pH, and the switch between fusogenic and nonfusogenic states occurs over a narrow pH range (White et al., 1983).

However, these results do not prove a role for low pH. We must caution that the effects of several additional factors, such as specific lipids, receptor binding, and transmembrane electrochemical gradients, must still be investigated. These factors may also alter the conformation or modify the effect of low pH.

What triggers the low-pH change? Electrostatic changes arising from protonation of acidic and/or basic groups could either break salt bridges, create charge repulsions, create isolated buried charges, alter hydrogen bonding, or increase hydrophobicity by neutralizing charges. One possibility that can be ruled out is that the *only* change at low pH is increased hydrophobicity due to neutralization of carboxyl groups. Because the pK_a's of such groups are lower in salt (Perrin & Dempsey, 1974), this would predict a salt-induced shift in transition pH, opposite in direction to that observed (Blewitt et al., 1984). Salt could affect the relative stability of the low-pH and neutral pH conformations through destabilization of salt bridges by competition, through changes in pK_a's of basic residues, or through destabilization of the compact protein conformation through salt effects on electrostatic repulsions.[7] The sharp low-pH change is most likely a cooperative process involving changes in several of these different types of interactions.

We note that the extensive aggregation of toxin at low pH is very important as it could give rise to various artifacts. In the experiments in this report, aggregation probably affects CD spectra and reversibility. In addition, if aggregation involves interaction of hydrophobic regions, it may compete with detergent binding, resulting in apparently weak detergent binding which could be mistaken for reduced hydrophobicity. These effects could explain the differences in toxin behavior at pH 3 and 4.2, although they could also result from real differences in conformation. Fluorescence intensity, λ_{max}, acrylamide quenching curves, and transition kinetics are all similar at pH 3 and 4.2. Therefore, these parameters are probably insensitive to aggregation. We are presently developing methods for more accurate determination of the degree of aggregation and for its control. We hope that a combination of biochemical and biophysical approaches will help us to understand the behavior of this protein.

Acknowledgments

We gratefully acknowledge the help of Reynold Homan and Moises Eisenberg in measurement of fluorescence lifetimes and the gift of purified toxin from Brian McKeever and Raghupathy Sarma.

References

Blewitt, M. G., Zhao, J.-M., McKeever, B., Sarma, R., & London, E. (1984) *Biochem. Biophys. Res. Commun. 120*, 286–290.

[6] J. Zhao and E. London, unpublished observations.

[7] This assumes the toxin is in a more extended conformation at low pH. In extended conformations, electrostatic repulsions between like-charged residues are more effectively shielded by salt-derived counterions because these ions can penetrate to the charged residues more effectively (Tanford, 1961). Therefore, salt increases the stability of an extended conformation relative to a compact one.

Boquet, P., Silverman, M. S., Pappenheimer, A. M., Jr., & Vernon, W. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4449–4453.

Bustamante, C., Tinoco, I., Jr., & Maestre, M. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3568–3572.

Chen, Y. H., Yang, J. T., & Chau, K. N. (1974) *Biochemistry 13*, 3350–3359.

Collier, R. J. (1982) in *ADP Ribosylation Reactions: Biology and Medicine* (Hayashi, O., & Ueda, K., Eds.) pp 575–592, Academic Press, New York.

Collier, R. J., & Kandel, J. (1971) *J. Biol. Chem. 246*, 1496–1503.

Collier, R. J., Westbrook, E. M., McKay, D. B., & Eisenberg, D. (1982) *J. Biol. Chem. 257*, 5283–5285.

Donovan, J. J., Simon, M. I., Draper, R. K., & Montal, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 172–176.

Draper, R. K., & Simon, M. I. (1980) *J. Cell Biol. 87*, 849–854.

Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672–679.

Eftnik, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–227.

Fasman, G. D., Ed. (1976) *CRC Handbook of Biochemistry and Molecular Biology: Physical and Chemical Data*, Vol. 1, CRC Press, Cleveland, OH.

Geisow, M. J. (1984) *Exp. Cell Res. 150*, 29–35.

Geisow, M. J., & Evans, W. H. (1984) *Exp. Cell Res. 150*, 36–46.

Gill, D. M., & Dinius, L. L. (1971) *J. Biol. Chem. 246*, 1485–1491.

Greenfield, L., Bjorn, M. J., Horn, G., Fong, D., Buck, G. A., Collier, R. J., & Kaplan, D. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6853–6867.

Greenfield, N. J., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.

Helenius, A., & Simons, K. (1972) *J. Biol. Chem. 247*, 3656–3661.

Huang, R. T. C., Rott, R., & Klenk, H.-D. (1981) *Virology 110*, 243–247.

Kaczorek, M., Delpeyroux, F., Chenciner, N., Streeck, R. E., Murphy, J. R., Boquet, P., & Tiollais, P. (1983) *Science (Washington, D.C.) 221*, 855–858.

Kagan, B. L., Finkelstein, A., & Colombini, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4950–4954.

Leese, R. A., & Wehry, E. L. (1978) *Anal. Chem. 50*, 1193–1197.

London, E., Zhao, J.-M., Chattopadhyay, A., Blewitt, M., McKeever, B., & Sarma, R. (1984) *Ann. N.Y. Acad. Sci. 435*, 558–560.

Maeda, T., & Ohnishi, S. (1980) *FEBS Lett. 122*, 283–287.

Manavalan, P., & Johnson, W. C., Jr. (1983) *Nature (London) 305*, 831–832.

Marnell, M. H., Shia, S.-P., Stookey, M., & Draper, R. K. (1984) *Infect. Immun. 44*, 145–150.

Marsh, M. (1984) *Biochem. J. 218*, 1–10.

Maxfield, F. R. (1982) *J. Cell Biol. 95*, 676–681.

McKeever, B., & Sarma, R. (1982) *J. Biol. Chem. 257*, 6923–6925.

Misler, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4320–4324.

Misler, S. (1984) *Biophys. J. 45*, 107–109.

O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Pappenheimer, A. M., Jr. (1977) *Annu. Rev. Biochem. 46*, 69–94.

Perrin, D. D., & Dempsey, B. (1974) *Buffers for pH and Metal Ion Control*, p 8, Chapman and Hall, London.

Proia, R. L., Eidels, L., & Hart, D. A. (1981) *J. Biol. Chem. 256*, 4991–4997.

Ratti, G., Rappuoli, R., & Giannini, G. (1983) *Nucleic Acids Res. 11*, 6589–6595.

Sandvig, K., & Olsnes, S. (1980) *J. Cell Biol. 87*, 828–832.

Sandvig, K., & Olsnes, S. (1981) *J. Biol. Chem. 256*, 9068–9076.

Simons, E. R. (1981) in *Spectroscopy in Biochemistry*, Vol. I, pp 63–153, CRC Press, Cleveland, OH.

Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp 472–473, Wiley, New York.

Torchinsky, Yu. M. (1981) *Sulfur in Proteins*, Pergamon Press, Oxford.

Tuzimura, K., Konno, T., Meguro, H., Hatano, M., Murakami, T., Kashiwabara, K., Saito, K., Kondo, Y., & Suzuki, T. M. (1977) *Anal. Biochem. 81*, 167–174.

Uchida, T. (1983) *Pharmacol. Ther. 19*, 107–122.

White, J., Matlin, K., & Helenius, A. (1981) *J. Cell Biol. 89*, 674–679.

White, J., Kielian, M., & Helenius, A. (1983) *Q. Rev. Biophys. 16*, 151–195.

Wilson, I. A., Skehel, J. J., & Wiley, D. C. (1981) *Nature (London) 289*, 366–373.