Comparison of the Conformation, Hydrophobicity, and Model Membrane Interactions of Diphtheria Toxin to Those of Formaldehyde-Treated Toxin (Diphtheria Toxoid): Formaldehyde Stabilization of the Native Conformation Inhibits Changes That Allow Membrane Insertion

Ranjana Paliwal and Erwin London*

Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, New York 11794-5215

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ABSTRACT: Toxoids are inactivated protein toxins that are used in vaccines. The behavior of diphtheria toxin reacted with formaldehyde (diphtheria toxoid) was compared to that of diphtheria toxin in order to understand the nature of the changes that occur in toxoids upon protein reaction with formaldehyde. Despite the intramolecular cross-links in the toxoid, the conformations of the toxoid and the toxin were very similar in both the native and low pH-induced membrane-penetrating states as judged by fluorescence and hydrophobicity properties. However, the toxoid underwent thermal-, low-pH-, and guanidinium chloride-induced conformational changes only at more extreme conditions than needed to induce such changes in the toxin. This implies that formaldehyde modification stabilizes the native conformation relative to several conformations that involve different degrees of unfolding. The stabilization to conformational changes induced by low pH is particularly interesting because low pH induces partial unfolding of the toxin to a molten globule-like state. It was found that the toxoid only gained the ability to interact with model membrane vesicles at a lower pH than the toxin. Because low-pH-induced unfolding and membrane interaction are critical steps in the entry of diphtheria toxin into cells, the resistance of the toxoid to these changes may be linked to its lack of toxicity. The implications of these results for the construction of toxoids are discussed.

Toxoids are inactivated protein toxins used in vaccines. Traditionally, they have been prepared by formaldehyde treatment. Modern molecular biological techniques have refocused efforts to develop toxoids through inactivation of toxin proteins via site-directed mutagenesis (Killeen et al., 1992; Pizza et al., 1988, 1994; Barbieri et al., 1992; Marsili et al., 1992). To understand the properties of a toxoid that might contribute to its inactivation, and the effects of formaldehyde cross-linking on proteins, the conformational and membrane-interacting properties of diphtheria toxoid were examined.

Diphtheria toxoid is the functional component of the vaccine used to prevent diphtheria. It is an inactivated form of diphtheria toxin. The toxin is a protein secreted by *Corynebacterium diphtheriae* as a single polypeptide. It can be split into two chains, A (M_r 21 167) and B (M_r 37 199), which are joined by a disulfide bond (Collier, 1982). The crystal structure of diphtheria toxin has been shown to consist of three domains (Choe et al., 1992). The catalytic domain is identical to the A chain. The B chain contains the transmembrane and receptor binding domains. The transmembrane domain is made up of nine α-helices. Several of these helices contain hydrophobic sequences which play a critical role in membrane insertion and translocation. The receptor binding domain is made up of a β-barrel. This domain binds the toxin to its receptor, which appears to be a complex of a membrane-anchored epidermal growth factor (EGF)-like protein with a second membrane protein (Iwamoto et al., 1991; Naglich et al., 1992).

Subsequent to receptor binding, the toxin enters the cells via receptor-mediated endocytosis. Membrane penetration is believed to occur after the toxin reaches endosomes (London, 1992a), triggered by low pH within the endosomal lumen (pH 5.3 within late endosomes; Fuchs et al., 1989). This low pH induces partial unfolding of the toxin, resulting in exposure of the hydrophobic regions within the B chain and translocation of the A chain of the toxin into the cytoplasm (London, 1992a). Once in the cytoplasm, the A chain catalyzes the transfer of the ADP ribosyl group of NAD^+ to elongation factor 2 (EF-2) (Collier, 1982). This inactivates EF-2, stopping protein synthesis and killing the target cell.

Diphtheria toxin is converted into toxoid by treatment with formaldehyde (Linggood et al., 1962). In this work, we have examined the behavior of the formaldehyde-modified protein in terms of the conformational changes that lead to membrane penetration. These latter changes may play a role in toxoid inactivation by inhibiting the ability of the toxoid to translocate across membranes and may have important implications for the design of medically useful toxoids.

EXPERIMENTAL PROCEDURES

Materials. Formaldehyde was obtained from Fisher Scientific as a 37% (w/w) solution. Gel electrophoresis supplies were purchased from Pharmacia. DOPC[1] and DOPG were purchased from Avanti Polar Lipids (Pelham, AL). Triethylammonium salts of the biotinylated lipids and

* Author to whom correspondence should be addressed. Phone: (516) 632-8564. Fax: (516) 632-8575. E-mail: elondon@ccmail.sunysb.edu.

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rhodamine-DHPE were purchased from Molecular Probes (Eugene, OR). The exact concentrations of the phospholipids were measured by phosphate analysis. Diphtheria antitoxin (20 000 units of purified concentrated equine globulin) was purchased from Connaught. Other Western blot reagents were purchased from Sigma. All other chemicals were reagent grade.

Preparation of Diphtheria Toxin and Toxoid. Diphtheria toxin was purchased from Connaught Laboratories (Ontario, Canada) and purified as previously described (Collier & Kandel, 1971; McKeever & Sarma, 1982; Tortorella et al., 1993). Purified free monomer toxin (2−10 mg/mL) was stored at 4 °C in 5 mM Tris-HCl, 1 mM EDTA, and 0.02% (w/v) sodium azide (pH 7.0). A 200 μg/mL stock solution of toxin was made by dilution of the purified toxin with 10 mM sodium phosphate and 150 mM NaCl (pH 7, PBS). To prepare toxoid, 200 μg of purified toxin was made up to 1 mL with PBS, to which was added either 2 or 4 μL of 37% (w/w) formaldehyde (i.e., a final concentration of 0.2 or 0.4% formalin, respectively), and then the solution incubated at room temperature for 4−5 days (Linggood et al., 1963). The toxoid was then stored in the presence of formaldehyde at room temperature.

In general, we found the behavior of the toxoid was similar in preparations made with different concentrations of formaldehyde. Usually, resistance to unfolding (see Results) was slightly greater with higher levels of formaldehyde, although some variability was noted in preparations even when using the same amounts of formaldehyde. Almost all of the experiments described in this report were also repeated using commercially prepared purified diphtheria toxoid (Connaught Laboratories). The properties of the commercial toxoid were very similar to those of our preparations.

Fluorescence Spectroscopy. Fluorescence was measured with a Spex 212 Fluorolog spectrofluorimeter using semimicro quartz cuvettes with a 1 cm excitation path length and a 4 mm emission path length. Generally, 2.5 mm slits were used for excitation and 5 mm slits for emission. This corresponds to nominal band-passes of 4.5 and 9 nm, respectively.

Fluorescence vs pH. Buffers (10 mM) were prepared with 150 mM NaCl. Sodium acetate buffer was used over the pH range 3.3−5.5 and sodium phosphate buffer for pH 6.0−7.0. Twenty-five microliters of 200 μg/mL toxin or toxoid was added to 1 mL of buffer at room temperature. Samples were then incubated for at least a few minutes before fluorescence was measured. Fluorescence emission spectra was measured from 315 to 365 nm, with the excitation wavelength set at 280 nm. Background spectra of the buffers were subtracted to obtain protein fluorescence. The final pH of each sample was also measured.

Fluorescence vs Gdm-Cl. A stock solution was prepared containing 8 M Gdm-Cl in 10 mM sodium phosphate and 150 mM NaCl, adjusted to pH 7.0. Various amounts of Gdm-Cl were diluted to 0.97 mL with PBS. Then, 25 μL of 200 μg/mL toxin or toxoid was added and fluorescence measured after at least 15 min of incubation. Fluorescence emission spectra were measured as described above. Background spectra of sample without protein but with Gdm-Cl were subtracted to obtain protein fluorescence.

Temperature Dependence of Fluorescence. Samples were prepared by addition of 25 μL of 200 μg/mL toxin or toxoid to 1 mL of 10 mM sodium acetate or sodium phosphate and 150 mM NaCl at various pH values. Samples of toxin were cooled to 19 °C, and then fluorescence was measured while heating to 60 °C at a rate of about 0.5 °C/min. Fluorescence emission was measured at 330 and 350 nm, with the excitation wavelength set at 280 nm. The background fluorescence was measured at low and high temperatures, found to be insignificant compared to protein fluorescence (2%), and not subtracted.

Acrylamide Quenching of Fluorescence. Samples containing 25 μL of 200 μg/mL toxin or toxoid were added to 1 mL of PBS (pH 7.0) or 10 mM sodium acetate and 150 mM NaCl (pH 4.0). Each sample was titrated with 10 μL aliquots of 5 M acrylamide dissolved in water, fluorescence was measured, and then the next aliquot of acrylamide was added. Fluorescence values were corrected for dilution by the acrylamide, inner filter effect due to acrylamide absorbance at 295 nm and background fluorescence in controls without protein. Intensity was measured at excitation (295 nm) and emission (340 nm).

Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) was done using a PhastSystem (Pharmacia LKB Biotechnology). Three volumes of a sample (3 or 6 μL) were added to one volume of 4× loading buffer [200 mM Tris-HCl, 4 mM EDTA, 10% (w/v) SDS, and 0.04% (w/v) bromphenol blue (pH 8)] or 4× reducing loading buffer (four volumes of 4× loading buffer plus one volume of β-mercaptoethanol). Samples were incubated at room temperature, run on 10−15% gradient gels, and visualized by Coomassie Blue staining (unless otherwise noted, nonreducing conditions were used.)

Hydrophobicity Analysis by Triton X-114 Phase Separation. The method of Bordier (1981) was used to measure toxin and toxoid interaction with detergent. A roughly 10% (v/v) solution of Triton X-114 in water (10 μL) was mixed with 100 μL of 10 mM sodium acetate and 150 mM NaCl (pH 3.6) or 10 mM sodium phosphate and 150 mM NaCl (pH 5.2). Six micrograms of 200 μg/mL toxin or toxoid was added and the mixture incubated at 37 °C for 30 min (final volume, 140 μL; final pH, 3.9 for acetate-containing samples and 5.9 for phosphate-containing samples). The mixture was pelleted in a table top centrifuge (10000*g*) for 10 min. The supernatant (100 μL) was removed, and 60 μL of the appropriate buffer was added to the pellet and remaining supernatant. The pure supernatant fraction or the diluted pellet-enriched fraction (6 μL) was then analyzed by SDS gel electrophoresis.

Preparation of Small Unilamellar Vesicles (SUV). SUV were prepared by sonication. A mixture (w/w) of 20% DOPG, 78% DOPC, 2% biotinylated-PE (1% biotin-DHPE and 1% biotin-X-DHPE), and approximately 0.25% rhodamine-DHPE was first dried under a stream of nitrogen and then further dried under high vacuum for 1 h. The dried lipids were suspended in 150 mM NaCl at a concentration of 20 mg/mL, deoxygenated with nitrogen, and then sonicated for 30−40 min using a bath sonicator (Laboratories Supply Co., Hicksville, NY) until nearly optically clear.

[1] Abbreviations: biotin-DHPE, *N*-biotinoyl-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; biotin-X-DHPE, *N*-[6-[(biotinoyl)-amino]hexanoyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; DMF, dimethylformamide; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; EDTA, disodium ethylenediaminetetracarboxylic acid; Gdm-Cl, guanidinium chloride; PBS, 10 mM sodium phosphate and 150 mM NaCl (pH 7.0); rhodamine-DHPE, *N*-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine.

Toxin and Toxoid Binding to SUV. SUV (20 μL) from the 20 mg/mL stock solution prepared as described above were added to 100 μL of 10 mM sodium acetate and 150 mM NaCl or 10 mM sodium phosphate and 150 mM NaCl at the appropriate pH. Fifty microliters of 200 μg/mL toxoid or 50 μL of PBS plus 2 μL of 5 mg/mL toxin were also added. The samples were then incubated for 30 min at 23 °C. To pellet the SUV, the method of Tortorella et al. (1993) was used. Twelve microliters of approximately 5 mg/mL streptavidin was added and incubated at 23 °C for 20 min. The sample was then centrifuged at 10000*g* for 30 min. The amount of SUV pelleting was checked in one set of samples by measuring pelleting of SUV-incorporated rhodamine-DHPE. Rhodamine-DHPE was detected by its fluorescence with emission at 590 nm and excitation at 570 nm. About 90% pelleting of SUV was obtained.

Both supernatant and pellet were analyzed for toxin or toxoid content by SDS gel electrophoresis followed by Western blotting using the PhastSystem. Samples were first run on a 10−15% SDS gel, and the gel was transferred onto nitrocellulose paper presoaked in 25 mM Tris, 192 mM glycine, and 20% methanol (transfer buffer) using the Pharmacia transfer system and directions. The nitrocellulose paper was then soaked in blocking buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% (v/v) Tween 20, 3% (w/v) nonfat dry milk, and 0.05% (w/v) sodium azide] for 30 min with shaking. Then the blot was incubated for 1 h in primary antibody solution [a 1/500 (v/v) dilution of diphtheria antitoxin in blocking buffer prepared from a stock of antitoxin that had been diluted to 20 mg/mL with water]. The nitrocellulose was then washed for 30−45 min in TBS−Tween [25 mM Tris, 150 mM NaCl (pH 7.4), and 0.2% (v/v) Tween 20) with shaking. The blot was then incubated for 1 h with rabbit anti-horse IgG conjugated to alkaline phosphatase (Sigma) diluted 1/500 (v/v) with blocking buffer, again followed by 30−45 min of washing in TBS−Tween. Protein bands were visualized in a mixture of 66 μL of 50 mg/mL nitroblue tetrazolium in 70% DMF, 33 μL of 50 mg/mL bromochloroindoylphosphate in 100% DMF, and 10 mL of 100 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$ (pH 9.5).

RESULTS

SDS Gel Analysis of Toxin and Toxoid. The exact degree of protein cross-linking by formaldehyde is difficult to analyze because it is chemically complex (see Discussion). Nevertheless, SDS gel electrophoresis can provide some information about cross-linking. Preparations of formaldehyde-treated toxin (which we will refer to as toxoid) and the toxin from which it was prepared are shown in Figure 1.[2] The toxoid runs with a mobility corresponding to a slightly lower apparent molecular weight than the whole toxin. This probably is due to intramolecular cross-linking within the toxoid causing it to take on a more compact conformation in SDS (see Discussion). The diffuse toxoid band may reflect the presence of toxoid variants with different degrees and/or sites of cross-linking. Under reducing conditions (lane 6), the toxin dissociated into A and B chains, indicating that, as usual, a large proportion of toxin molecules were nicked between the A and B chains. In contrast, the toxoid prepared from this toxin was resistant to dissociation by reduction (lanes 2 and 4), indicating that in the toxoid the A and B chains are cross-linked. An alternate possibility is that cross-linking in the toxoid buries the disulfide bond linking the A and B chains, making it inaccessible to reduction. This is unlikely because the disulfide linking A and B chains is accessible to reduction even in the folded native conformation (Collier & Kandel, 1971).

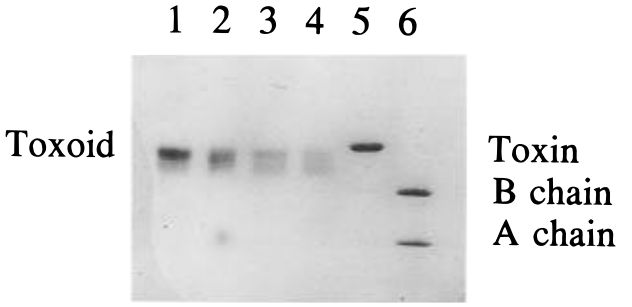

FIGURE 1: SDS−PAGE analysis of the toxin and toxoid preparations in the absence (lanes 1, 3, and 5) and in the presence (lanes 2, 4, and 6) of the reducing agent β-mercaptoethanol. Lanes 1 and 2: a 0.074% formaldehyde (0.2% formalin) preparation of toxoid. Lanes 3 and 4: a 0.148% formaldehyde (0.4% formalin) preparation of toxoid. Lanes 5 and 6: diphtheria toxin without formaldehyde treatment.

The electrophoretic behavior of the toxoid was also examined after incubation under more extreme conditions. There was no change in the differences between the mobility of toxin and toxoid (prepared with 0.2% formalin) on nonreducing gels after 1 h of preincubation of 60 μg/mL toxoid at pH 4, pH 7 at 60 °C, or pH 7 in 3.7 M Gdm-Cl (not shown). This implies that under these conditions there was no significant reversal of formaldehyde cross-linking.

Fluorescence of Diphtheria Toxin and Toxoid vs pH. Diphtheria toxin undergoes a functionally important conformational change to a partly unfolded form at low pH (London, 1992a). In order to compare toxoid and toxin conformations at different pH values, protein fluorescence was measured (Blewitt et al., 1985). Figure 2A compares the effect of pH on the intrinsic fluorescence intensity of diphtheria toxin and toxoid. In agreement with our previous results, in the toxin, Trp emission shows a sharp transition in intensity with a midpoint at pH 5, and weaker intensity at low pH (Blewitt et al., 1985). A similar transition is seen for the toxoid. However, the transition occurs at a lower pH, with a midpoint close to pH 4.2.

The emission spectra of toxin and toxoid were also measured. Shifts in Trp emission toward longer wavelengths are correlated with a change to a more polar Trp environment. The ratio of emission at 330 nm to that at 350 nm is a sensitive measure of such a shift, with an increase in this ratio corresponding to a shift to longer wavelengths (Jiang et al., 1991). Figure 2B shows that the toxin and toxoid Trp are in similar environments at neutral pH. At low pH, Trp emission shifts to longer wavelengths for both the toxin and toxoid, although slightly less so for the toxoid. As in the case of fluorescence intensity, the change in toxoid emission occurs at a lower pH than for the toxin.

These properties suggest that the toxin and toxoid have similar conformations, but the difference in the transition pH shows that the native state is stabilized relative to the

[2] It should be pointed out that, although the level of cross-linking in our preparations may differ a small amount from that in commercial toxoid preparations due to slight variations in toxoiding protocols (Linggood et al., 1963; Pappenheimer et al., 1972; Stainer, 1968), we obtained almost identical results in studies in which commercially prepared diphtheria toxoid was used (not shown).

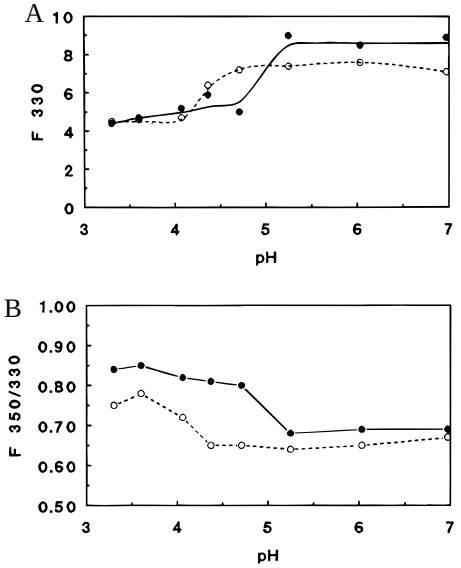

FIGURE 2: Effect of pH on the tryptophan fluorescence emission of diphtheria toxoid and toxin. Samples contained 5 μg/mL toxin or 0.2% formalin toxoid. (Top) Intensity of toxin (●) or toxoid (○) emission at 330 nm (relative units). (Bottom) The ratio of fluorescence emission of toxin (●) or toxoid (○) at 350 nm to that at 330 nm.

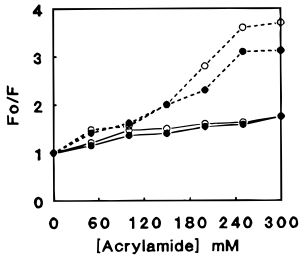

FIGURE 3: Acrylamide quenching of toxin and toxoid tryptophan fluorescence. Samples contained 5 μg/mL toxin or 5 μg/mL 0.2% formalin toxoid. The ratio of initial fluorescence in the absence of acrylamide (F_0) to the fluorescence in the presence of various concentrations of acrylamide (F) is shown vs acrylamide concentration for the (●) toxin or (○) toxoid at pH 4.0 (- - -) and the toxin (●) and toxoid (○) at pH 7.0 (—).

low-pH form in the case of the toxoid. The smaller shift in toxoid fluorescence at low pH suggests that toxoid Trp may be slightly less exposed than in the toxin, consistent with a slightly lesser degree of low-pH-induced unfolding in the toxoid, and the more compact conformation for SDS-unfolded toxoid (see above).

Acrylamide Quenching of Toxin and Toxoid Trp Fluorescence. Acrylamide is a soluble fluorescence quencher that tests the average exposure of Trp residues in protein to aqueous medium (Eftink & Ghiron, 1981). A higher exposure of Trp residues to the aqueous medium gives rise to stronger quenching. Acrylamide quenching was used to confirm the effect of pH upon exposure of Trp residues in toxin and toxoid.

Figure 3 shows a graph of F_0/F, which is the ratio of Trp fluorescence in the absence of acrylamide to that in the presence of acrylamide, vs acrylamide concentration. The pH dependence of acrylamide quenching of toxin fluorescence is similar to that seen previously (Blewitt et al., 1985). Acrylamide quenching of the toxin and toxoid is very similar. There is increased acrylamide quenching at pH 4 relative to that at pH 7 in both cases. This further supports the observation that the toxin and toxoid have similar native and low-pH-induced conformations.

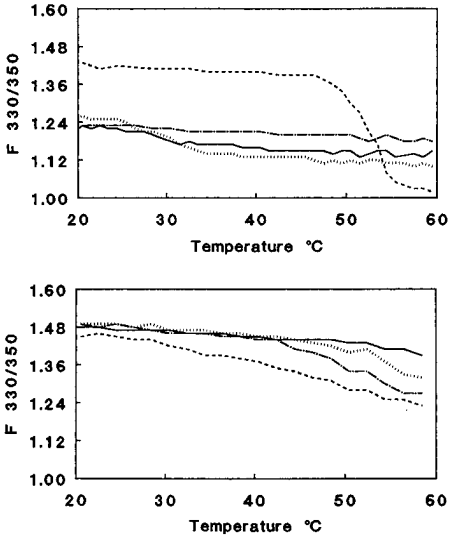

FIGURE 4: Effect of temperature on the fluorescence of diphtheria toxin and toxoid at various pHs. Samples contained 5 μg/mL toxin or 5 μg/mL 0.4% formalin toxoid. The temperature was increased at a rate of 0.5 °C/min, and the ratio of emission at 330 nm to that at 350 nm was measured every 1 °C. (Top) Toxin at (- - -) pH 7, (···) pH 4.6, (—) pH 4.3, and (—·—) pH 4.0. (Bottom) Toxoid at (—) pH 5, (···) pH 4.8, (—·—) pH 4.6, and (- - -) pH 4.3. The effect of temperature on toxoid fluorescence at pH 7 is similar to that at pH 5.

Effect of Temperature on the Conformation of Toxin and Toxoid. The thermal stabilities of the native conformations of toxin and toxoid were also compared. Thermal unfolding was followed by the increased exposure of toxin Trp residues that occurs in the thermally denatured state (Zhao & London, 1986). This was monitored via fluorescence via the 330 nm/350 nm emission ratio. Figure 4 shows the dependence of the emission ratio on temperature. For the toxin, a distinct unfolding transition is observed with a midpoint (T_m) near 52 °C at pH 7, in agreement with previous results (Zhao & London, 1986). This unfolding is characterized by a distinct red shift in emission. Below pH 5, the toxin is at least partly unfolded at all temperatures examined, again in agreement with previous results (Zhao & London, 1986).[3] In contrast, the toxoid is significantly stabilized against thermal unfolding relative to the toxin. Except at the lowest pH, T_m is very high, probably well above 60 °C at pH 5.

Unfolding of Toxin and Toxoid by Gdm-Cl. Unfolding of toxin and toxoid by Gdm-Cl was also compared. Previous studies have shown Gdm-Cl is able to completely unfold diphtheria toxin (Zhao & London, 1986; Jiang et al., 1991). In agreement with these studies, Figure 5 shows that, upon unfolding of diphtheria toxin, a large decrease in Trp fluorescence and a shift in Trp emission maximum to much longer wavelengths are observed (Jiang et al., 1991). Figure 5 shows that the toxoid also undergoes Gdm-Cl-induced unfolding, with similar changes in Trp fluorescence. However, the toxoid is more resistant to unfolding, which is 50% complete at about 2 M Gdm-Cl, in contrast to only 1 M for the toxin. The slightly smaller wavelength shift seen in the toxoid even at the highest Gdm-Cl concentrations suggests the toxoid is less completely unfolded than the toxin, at least in the vicinity of the Trp residues.

Hydrophobicity of Diphtheria Toxin and Toxoid. Previous studies have shown that low-pH-induced conformational

[3] The residual transition seen near 30 °C is due to unfolding of the A chain (Ramsay et al., 1988).

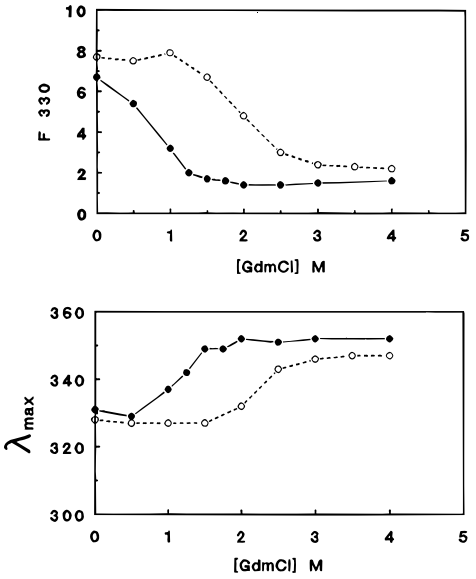

FIGURE 5: Effect of guanidinium chloride on the fluorescence of diphtheria toxin and toxoid. Samples contained 5 μg/mL toxin or 5 μg/mL 0.2% formalin toxoid at pH 7. (Top) Intensity of toxin (●) or toxoid (○) emission at 330 nm. (Bottom) Wavelength of maximum fluorescence emission of toxin (●) or toxoid (○).

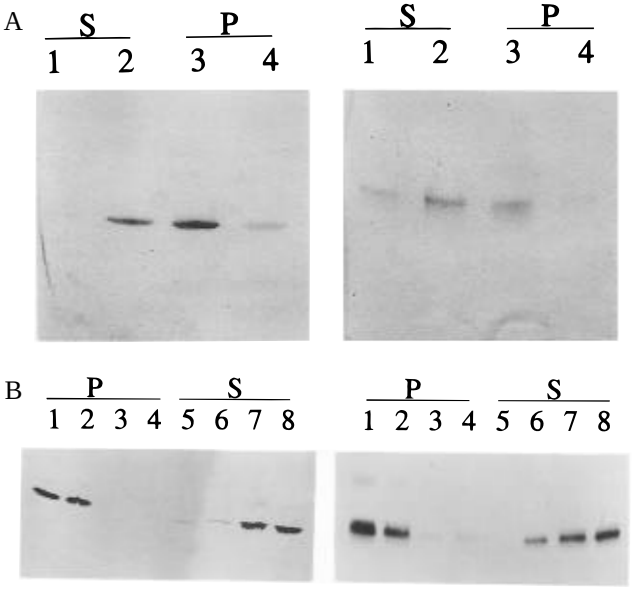

FIGURE 6: Effects of pH on toxin and toxoid interaction with micelles and model membranes. (A, Top) SDS−PAGE analysis of the supernatant and pellet obtained after Triton X-114 phase separation of the diphtheria toxin and 0.2% formalin toxoid. Lane 1: pH 3.9 supernatant. Lane 2: pH 5.9 supernatant. Lane 3: pH 3.9 pellet-enriched fraction. Lane 4: pH 5.9 pellet-enriched fraction. (Left) Diphtheria toxin. (Right) Diphtheria toxoid. (B, Bottom) Western blot analysis of the association of toxin and toxoid binding to SUV. Blots show supernatant and pellet obtained after incubation of protein with SUV and pelleting of SUV and SUV-bound protein. Lanes 1−4: pellet at pH 4.0, 4.6, 6.0, and 7.0, respectively. Lanes 5−8: supernatants at pH 4.0, 4.6, 6.0, and 7.0, respectively. (Left) Diphtheria toxin. (Right) Formalin toxoid (0.2%). Results very similar to B were obtained with 0.4% formalin toxoid.

change renders diphtheria toxin hydrophobic and triggers membrane insertion [reviewed in London (1992a)]. Triton X-114 phase separation was used to compare the hydrophobicities of the toxin and toxoid (Figure 6A). In this method, hydrophobic proteins are recovered in a detergent phase which is separated as a pellet upon centrifugation, while hydrophilic proteins remain in the aqueous supernatant (Bordier, 1981). At the higher pH, toxin appears in the aqueous phase, and at the lower pH, it appears in the detergent phase,[4] consistent with previous studies showing that the toxin is hydrophobic only at low pH. Figure 6A shows similar behavior for the toxoid, indicating that the toxoid also becomes hydrophobic at low pH, although the appearance of a small amount of toxoid in the aqueous phase at pH 3.9 hints that it may be slightly less hydrophobic than the toxin at this pH. Control experiments showed that there was no pelleting of toxin at low pH in the absence of Triton X-114.

Binding of Toxin and Toxoid to Small Unilamellar Model Membrane Vesicles. The binding of toxoid and toxin to small unilamellar vesicles (SUV) was examined at various pH values to see if they shared similar lipid binding properties. This was done by pelleting SUV in the presence of toxin or toxoid and measuring the amount of bound protein. Western blot analysis of toxin and toxoid bound to SUV is shown in Figure 6B. The toxin is totally associated with SUV at low pH below 5. This is consistent with previous studies of the toxin−vesicle association which show that only the low-pH conformation binds vesicles (Hu & Holmes, 1984; Chung & London, 1988). The toxoid also gains the ability to bind to SUV at low pH. However, the association of the toxoid with SUV requires lower pH, being only about half complete at pH 4.6.[5] This is consistent with the observation that the conformational change in the toxoid occurs at a lower pH than that in the toxin.

DISCUSSION

How Formaldehyde Modification of Diphtheria Toxin Would Inhibit Its Activity. This report shows that formaldehyde modification of toxin results in (1) the stabilization of the native structure of diphtheria toxin, such that it fails to undergo the low-pH-induced conformational change at endosomal pH, and (2) the prevention of the dissociation of toxin into A and B chains upon reduction. Both of these changes would contribute significantly to the inactivation of the toxin. The first would inhibit formation of the membrane-inserting hydrophobic conformation under endosomal conditions. A number of studies have established that the unfolding of diphtheria toxin at low pH is the key step in exposing hydrophobic sites on the toxin and triggering its insertion and membrane penetration [reviewed in London (1992a,b)]. Unfolding at low pH, critical for translocation, may occur in both the A chain and the B chain (Zhao & London, 1988; Zhan et al., 1994; Falnes et al., 1994; Tortorella & London, 1995a,b). The second effect of formaldehyde, cross-linking of the A and B chains, would prevent the A chain from entering the cytoplasm, as it would remain permanently linked to the membrane-inserted B chain. Since ADP ribosylation activity requires dissociation of the A and B chains, it would also be inhibited by cross-linking

[4] The toxin in the pellet fraction at pH 5.9 is due to carryover of supernatant (see Experimental Procedures). There is also some carryover of toxoid in the supernatant at pH 5.9, but it is harder to see due to the diffuse nature of the toxoid band.

[5] The toxin appears to insert at a slightly higher pH than that which induces the conformational transition in the absence of lipid due to an effect of lipid on the conformational transition pH. Since lipid interacts most strongly with the low-pH-induced conformation, it shifts the equilibrium between native and low-pH conformations toward the latter form. This results in an upward shift in the transition pH (Chung & London, 1988).

(Collier et al., 1982). Therefore, these changes should contribute to the inability of toxoid to attack cells. This does not imply that these are the only effects of formaldehyde modification. We have not tested whether the toxoid retains the ability to bind to its receptor. Also, formaldehyde may directly block substrate binding to the active site. In support of this, we have found (not shown) that the nucleotide-free toxoid is unable to bind to columns containing triazinyl ligands mimicking NAD^+ (Carroll et al., 1986).

These observations may have implications for preparation of toxoids. Toxoids can be prepared simply by inactivation of the catalytic activity of a toxin (Killeen et al., 1992; Pizza et al., 1988, 1994; Barbieri et al., 1992; Marsili et al., 1992). However, when a toxin is catalytically inactivated by site-directed mutagenesis to prepare a toxoid, it will not duplicate all of the changes that occur upon formaldehyde cross-linking. In some cases, combining the effects of a catalytic mutation with formaldehyde treatment may yield a more effective toxoid (Rappuoli, 1994).

Effect of Formaldehyde Modification on Toxin Unfolding. The reaction between formaldehyde and proteins is complex. Formaldehyde reacts readily with various functional groups, but especially the functional groups of lysine and cysteine. In diphtheria toxoid, at least 30% of ϵ-amino groups can be blocked, so modification can be quite extensive (Blass et al., 1967). Reaction of the modified lysine residues to glutamine, asparagine, arginine, and tyrosine then results in cross-linking (Fox et al., 1985; Kunkel et al., 1981). It is most likely that multiple formaldehyde-induced cross-links is the factor stabilizing toxoid folding, because ordinary modification of amino acid side chains tends to destabilize protein folding. However, we cannot rule out effects due to other formaldehyde modifications. For example, the loss of charge on Lys residues due to formaldehyde modification would reduce the positive charge on the toxin, and this in turn could reduce electrostatic repulsions that contribute to unfolding (London, 1992a) and electrostatic effects that contribute to interaction with lipid (Chung & London, 1988).

Formaldehyde-induced stabilization of folding is not surprising in view of the use of aldehydes to fix biological structures in their original state and previous studies showing formaldehyde modification often does not significantly alter protein secondary structure (Mason & O'Leary, 1991). It is particularly interesting that formaldehyde cross-linking induces resistance of the toxoid to low-pH-induced conformational changes, because at low pH diphtheria and other toxins take on a molten globule-like conformation (van der Goot et al., 1991; London, 1992b). The nature of the molten globule state is an area of intense research because of its apparent role in protein folding and unfolding processes (Bychkova et al., 1992; Ewbank & Creighton, 1991; Martin et al., 1991). In the molten globule, it is believed that tertiary structure is disrupted. Thus, our observations suggest that formaldehyde modification can inhibit the structural changes needed to form the molten globule.

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