Folding Changes in Membrane-Inserted Diphtheria Toxin That May Play Important Roles in Its Translocation†

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ABSTRACT: Diphtheria toxin membrane penetration is triggered by the low pH within the endosome lumen. Subsequent exposure to the neutral pH of the cytoplasm is believed to aid in translocation of the catalytic A domain of the toxin into the cytoplasm. To understand the effects of low pH and subsequent exposure to neutral pH on translocation, we studied toxin conformation in solution and in toxin inserted in model membranes. Two conformations were found at low pH. One form, L′, predominates below 25–30 °C, and the other, L″, predominates above 25–30 °C and is formed from the L′ state by an unfolding event. Both forms are hydrophobic and penetrate deeply into membranes. After pH neutralization, the L′ and L″ conformations give rise to two new conformations, R′ and R″, respectively. The R′ and R″ conformations differ from each other in that in the R′ state the A domain remains folded, whereas in the R″ state the A domain is unfolded. This is confirmed by the finding that only the R′ state possesses the capacity to bind and hydrolyze NAD^+. It is also supported by the finding that the R″ state can also be formed by thermal unfolding of the R′ state. The R conformations differ from the low-pH L conformations in that although they remain largely membrane-inserted, it appears that a large portion of the toxin is no longer in contact with the hydrophobic core of the bilayer. Since the two different R states arise from the two different L states, it is likely that the L′ state contains folded A domain and the L″ conformation contains unfolded A domain. It has also been found that after pH neutralization the release of the A domain by disulfide reduction of membrane-inserted toxin can result in A domain refolding. On the basis of this result and the finding that the less folded L″ and R″ forms of the toxin predominate at 37 °C, we conclude that the behavior of the toxin at physiological temperatures is consistent with translocation models proposing an unfolding/refolding cycle.

Diphtheria toxin is a protein (M_r 58 348) secreted by *Corynebacterium diphtheriae*. It can be readily cleaved by proteolysis into two fragments or domains, a relatively hydrophilic one called A (M_r 21 167) and a more hydrophobic one called B (M_r 37 199), which are joined by a disulfide bond (Pappenheimer, 1977; Collier, 1982; Murphy, 1985; Greenfield et al., 1983). Evidence suggests that after endocytosis the toxin enters the cytoplasm via penetration through the membrane of an acidic organelle (Draper & Simon, 1980; Sandvig & Olsnes, 1980, 1981; Marnell et al., 1984). Penetration is at least partially triggered by a low-pH-induced conformational change in the toxin (Sandvig & Olsnes, 1981; Donovan et al., 1981; Kagan et al., 1981; Hu & Holmes, 1984; Blewitt et al., 1984). After membrane penetration, domain A is translocated across the membrane and released into the cytoplasm where it inhibits protein synthesis by catalyzing the transfer of the ADP-ribose moiety of NAD^+ onto elongation factor 2 (Pappenheimer, 1977; Collier, 1982). It has also been suggested the toxin may induce internucleosomal breakdown (Chang et al., 1989).

The toxin represents a simple system for which transmembrane translocation of a protein can be studied (Olsnes et al., 1988) and may yield important general implications for understanding the membrane translocation of cellular proteins (for example, being unfolded is important for translocation of both toxin and ordinary cellular proteins (Eilers & Schatz, 1988; Liu et al., 1988; and see below)). Toxin translocation may also provide critical information for the design of therapeutically useful immunotoxins, an area of intense research (Collier & Kaplan, 1984; Williams et al., 1990). Several mechanisms for toxin translocation have been suggested. One model arises from the observation that toxin and its isolated B domain will induce pore formation in membranes at low pH (Donovan et al., 1981; Kagan et al., 1981; Shiver & Donovan, 1987; Deleers et al., 1983; Sandvig & Olsnes, 1988; Papini et al., 1988; Stenmark et al., 1989). This has raised the possibility that the A domain passes into the cytoplasm via this pore, perhaps in an unfolded form. It has also been suggested that in the membrane the hydrophobic B domain could act as a wrapper around domain A, with pore formation resulting subsequent to the release of the A domain (Misler, 1984).

On the other hand, photo-cross-linking, fluorescence, and membrane leakage studies on whole toxin and isolated domain A have demonstrated that domain A itself can interact with the lipid bilayer (Hu & Holmes, 1984; Zalman & Wisnieski, 1985; Montecucco et al., 1988; Zhao & London, 1988a; Jiang et al., 1989; Papini et al., 1987a,b). It has been proposed that the B domain only partially protects domain A from the bilayer (Ward, 1987; Papini et al., 1987b; Zhao & London, 1988a) in a cleft (Papini et al., 1987b) or partial wrapper (Zhao & London, 1988a). Pore formation may occur subsequently when the B domain closes around the gap left by domain A release (Papini et al., 1988; Zhao & London, 1988a). It should be noted that another possibility for the translocation mechanism is that toxin, which can destabilize bilayers (Cabiaux et al., 1984, 1985; Defrise-Quertain, 1989), may escape endosomes via membrane lysis (Hudson & Neville, 1985).

In previous studies, we have demonstrated the important role that low-pH-triggered denaturation-like processes play in the membrane insertion step. There we showed that it is

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the low-pH-induced partial unfolding of both whole toxin and isolated domain A that results in their becoming hydrophobic and interacting with membranes (Blewitt et al., 1984, 1985; Zhao & London, 1986, 1988a). We have proposed that partial unfolding of domain A is probably important for translocation.

In this report, we have studied the degree of folding and enzymatic activity of the toxin after low-pH treatment. Two conformations are found at low pH, and two different but related conformations are found after pH neutralization. These conformations differ in their folding and location in the membrane and yield important clues as to the role of folding changes in translocation.

Experimental Procedures

Materials. Partially purified diphtheria toxin was purchased from Connaught Laboratories (Ontario, Canada) and purified essentially as previously described (Collier & Kandel, 1971; McKeever & Sarma, 1982; Carroll et al., 1986). Free (i.e., without bound A_pU_p[1]) monomers were used in most experiments. This preparation contained a mixture of molecules nicked between the A and B domains and intact toxin as judged by SDS gel electrophoresis. Purified toxin was stored at 4 °C in 5 mM Tris/1 mM EDTA/0.02% (w/v) sodium azide, pH 7. Dioleoylphosphatidylglycerol, egg phosphatidylcholine, dioleoylphosphatidylcholine, 1-palmitoyl-2-(5-doxylstearoyl)phosphatidylcholine, and 1-palmitoyl-2-(12-doxylstearoyl)phosphatidylcholine were purchased from Avanti Polar Lipids (Pelham, AL). Lipid purity was confirmed, and concentrations were measured as described previously (Zhao & London, 1988a; Chattopadhyay & London, 1987). Dithiothreitol was purchased from Bio-Rad. Trypsin, A_pU_p, NAD^+, TLCK, and 2-mercaptoethanol were purchased from Sigma. [4-^{3}H]NAD^+ (1.6 Ci/mmol) was purchased from Amersham.

Model Membrane Preparation. Small unilamellar vesicles (SUV) were prepared by sonication except in the case of the spin-label experiments in which ethanol injection (Kremer et al., 1977) was used to conserve spin-labeled lipid. For sonication, mixtures of egg PC and PG in $CHCl_3$ were first dried under a stream of N_2 and then further dried under high vacuum for at least 1 h. The dried lipids were suspended in 150 mM NaCl at a concentration of 10 mg/mL, deoxygenated with N_2, and then sonicated in an argon-flushed tube for 30–60 min with a bath sonicator (Laboratories Supply Co., Hicksville, NY) until nearly optically clear. The sonicated vesicles were stored under argon at room temperature if they were kept overnight prior to use. For ethanol injection, a total of 320 nmol of a mixture of dioleoyl-PC, spin-labeled PC, and PG was dissolved in 15 μL of ethanol and subsequently diluted 100-fold in 10 mM sodium acetate/150 mM NaCl, pH 4.3. We found that 1% (v/v) ethanol in such samples did not affect toxin fluorescence properties and thermal transitions.

Fluorescence Spectroscopy. Fluorescence was measured with a Spex 212 Fluorolog spectrofluorometer using 1-cm path length quartz cuvettes. Unless otherwise noted, fluorescence intensity was measured at 280-nm excitation and 330-nm emission. Background intensity in samples without protein was subtracted. In general, fluorescence samples contained a final concentration of about 14 μg/mL toxin in buffer with 149 mM NaCl and in some samples with 0.2 mg/mL SUV.

For measurement of the temperature dependence of fluorescence, samples were prepared by adding 10 μL of 4.2 mg/mL toxin in 13.1 mM Tris-HCl/122 mM NaCl, pH 7, to a mixture of 12 μL of 200 mM sodium acetate, pH 4.38, and 60 μL of either 10 mg/mL 28% PG/72% PC (w/w) sonicated SUV in 150 mM NaCl or 150 mM NaCl, at either 23 or 37 °C, to obtain a final pH of 4.5. Samples were then incubated for 30 min. Where desired, the pH was then adjusted back to neutrality (pH 7–7.2) by the addition of 12 μL of 200 mM sodium phosphate, pH 8.2, and incubated an additional 30 min at the desired temperature. An aliquot containing 28 μg of toxin was then made up to 2 mL by the addition of 10 mM buffer and 150 mM NaCl at the desired temperature. The buffers used here were sodium acetate at pH 4.5 or phosphate at pH 7. For determination of the effect of reducing the disulfide link between A and B domains, 11.8 μL of either 100 mg/mL DTT or water was added 30 min after the neutralization of the above described mixture (made at 37 °C) and the mixture was incubated for an additional 30 min at 37 °C. Then an aliquot containing 28 μg of toxin was made up to 2 mL with 10 mM sodium phosphate/150 mM NaCl, pH 7, and incubated 30 min more before fluorescence was measured. In general, samples were prepared at room temperature (or 37 °C for the DTT experiments) and placed in a cell holder under thermostatic control at the starting temperature and the temperature setting was increased at 2–4 °C intervals, with a 15-min wait after each setting change to let sample temperature equilibrate. Actual sample temperature was measured with a digital thermometer in a cuvette containing water placed in the same sample holder. The buffers used had a negligible dependence of pH on temperature (Zhao & London, 1986).

For uncorrected fluorescence emission spectra, 400 μL of 0.07 mg/mL toxin in 15 mM Tris-HCl/150 mM NaCl, pH 7, was added to 40 μL of 10 mg/mL 28% PG/72% PC (w/w) sonicated SUV in 150 mM NaCl to give the spectrum of native state toxin.[2] Then, 10 μL of 200 mM sodium acetate, pH 4, was added to bring the pH to 4.5, and after a 30-min incubation at either 23 or 37 °C emission spectra were collected. To these samples was added a further 40 μL of 200 mM Na_2HPO_4 to bring the pH to 7–7.2, and after a 30-min incubation at the same temperature fluorescence was again measured. A final emission spectrum was collected after the pH-neutralized sample prepared at 23 °C was incubated for 30 min at 45 °C. Spectra shown have been corrected for dilution. In fluorescence quenching experiments, 15 μL of 1.4 mg/mL toxin in 5 mM Tris-HCl, pH 7, was added to 1.5 mL of the 2.1 mM 28% PG/72% PC (mol/mol) ethanol injection SUV described above. pH was neutralized to 6.9 by addition of 42 μL of 0.57 M Na_2HPO_4. Some quenching experiments were done starting at pH 4.5 and gave similar results to those done with pH 4.3 buffer.

Proteolytic Digestion and Gel Electrophoresis. Samples contained 4 μL of 1.05 mg/mL toxin in a solution of 14.5 mM Tris-HCl, 143 mM NaCl, pH 7, 6 μL of either 150 mM NaCl or 10 mg/mL sonicated SUV in 150 mM NaCl, and 1 μl of 200 mM sodium acetate, pH 4.38 (to yield pH 4.5), and were incubated for 30 min at the desired temperature. To reverse the pH to 7–7.2, we added, to samples that had been preincubated at low pH, 1 μL of 200 mM sodium phosphate, pH

[1] Abbreviations: A_pU_p, adenylyl(3′,5′)uridine 3′-phosphate; DTT, dithiothreitol; PC, diacyl-*sn*-glycero-3-phosphocholine; PG, dioleoyl-*sn*-glycero-3-phosphoglycerol; SUV, small unilamellar vesicles; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone.

[2] Since increased temperature decreases fluorescence intensity nonspecifically, spectra were all taken at 23 °C to allow direct comparisons. This can be done because the thermally induced conformational changes are irreversible (see text). It should be noted that since uncorrected spectra were measured, the emission λ_{max} is 2–3 nm higher than that observed with corrected spectra (Blewitt et al., 1984; Chung & London, 1988).

8.2, and then incubated for 30 min at the desired temperature. Some samples prepared at 23 °C were then incubated at 45 °C for 30 min. In samples in which ligand effects were examined, 1 μL of either 10 mM NAD^+ or water was then added. All samples were then incubated for 30 min more at 23 °C unless otherwise noted. To initiate digestion, 1 μL of freshly prepared trypsin was added. After the desired digestion interval at 23 °C, 1 μL of 40 mg/mL TLCK was added. Five minutes later, 4 μL of SDS gel loading buffer containing 200 mM Tris-HCl, pH 8, 4 mM Na_2EDTA, 10% (w/v) SDS, 0.04% (w/v) bromphenol blue, and 15% (v/v) 2-mercaptoethanol was added. After at least 5 min of incubation, 1 μL of the sample was analyzed by SDS gel electrophoresis by use of a Phastsystem (Pharmacia LKB Biotechnology). Gels with 10–15% gradients were used and protein bands visualized by Coomassie Blue staining (Heukeshoven & Dernick, 1988). We would like to point out that the total ionic strength varied somewhat among the different fluorescence and proteolysis samples. The total salt concentration was always well within a factor of 2 of 150 mM and therefore should not be a significant variable.

NAD^+ Glycohydrolase Activity. To prepare low-pH-treated samples, 15 μL of 4.2 mg/mL toxin was added to a mixture of 18 μL of 200 mM sodium acetate, pH 4.38, and either 90 μL of 10 mg/mL 28% PG/72% PC (w/w) sonicated SUV in 150 mM NaCl or 90 μL of 150 mM NaCl. The samples were then incubated at the desired temperature for 30 min, the pH was then adjusted to 7 by addition of 18 μL of 200 mM sodium phosphate, pH 8.2, and the samples were incubated for 30 min more. Some samples prepared at 23 °C were then incubated at 45 °C for 30 min. Samples prepared at higher temperatures were then incubated at 23 °C for 30 min more with the exception of those containing DTT. For DTT treatment, 11.8 μL of 100 mg/mL DTT was added to samples prepared at 37 °C and then the samples were incubated for 30 min at 37 °C. In all samples, the next step was the removal of an aliquot containing 14 μg of toxin for an activity measurement. Samples of toxin in the native state were prepared as in the above procedures except that 33 μL of 15 mM Tris-HCl, pH 7, was substituted for the sodium acetate and sodium phosphate. Activity was measured essentially by the method of Lory et al. (1980) in a total volume of 100 μL containing 58 mM sodium phosphate, pH 7.0, 50 μM NAD^+ (0.5 μM [^{3}H]NAD^+ and 49.5 μM nonradioactive NAD^+), and 140 μg/mL toxin. After incubation for 0, 60 or 120 min, hydrolyzed nicotinamide was immediately extracted by vigorous vortexing with 1 mL of water-satured ethyl acetate. The radioactivity of the released nicotinamide, in a 0.75-mL aliquot from the upper phase, was measured by scintillation counting. Radioactivity in control samples containing no toxin was subtracted to determine the level of hydrolysis.

Results

Temperature Dependence of the Conformation of Diphtheria Toxin at Low pH: Fluorescence Measurements. To characterize the temperature dependence of the degree of folding of the toxin after exposure to low pH, intrinsic toxin Trp fluorescence was measured (Figure 1). Shifts in emission maximum were generally measured by the ratio of intensity at 330 nm to that at 350 nm. This parameter is more sensitive to small wavelength shifts than λ_{max} (Jiang & London, 1990; Wharton et al., 1988). Emission spectra and λ_{max} values for the toxin are shown in Figure 1 (inset).

As shown in Figure 1 (left), at pH 4.5 heating toxin induces a fluorescence transition with a midpoint near 26 °C. This transition can also be detected by the decrease in overall

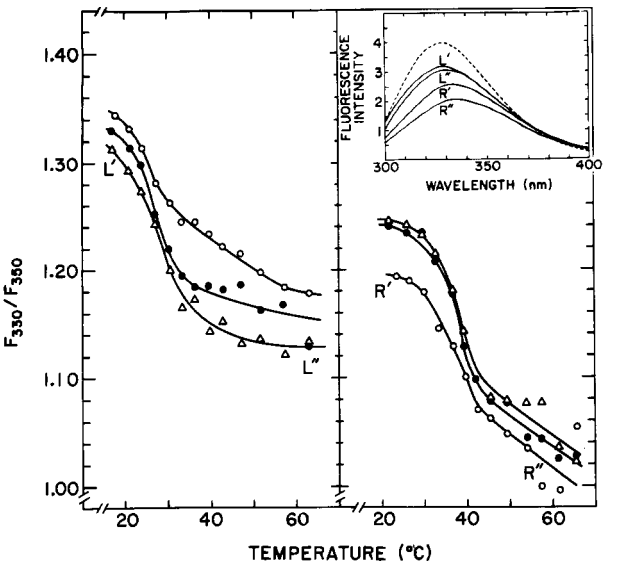

FIGURE 1: Effect of increasing temperature upon the fluorescence of low-pH-treated diphtheria toxin. Emission shifts were monitored by the ratio of fluorescence intensity at 330 nm to that at 350 nm (see text). The left panel shows the fluorescence of samples incubated at pH 4.5. The right panel shows the fluorescence of samples preincubated at pH 4.5 and then adjusted to pH 7–7.2 by the addition of sodium phosphate (see Experimental Procedures). Samples were prepared in the absence of lipid vesicles (△) or in the presence of 0.2 mg/mL SUV composed of (●) PC or (○) 28% PG/72% PC (w/w). The inset shows the fluorescence emission spectra of toxin in different conformational states in the presence of 28% PG/72% PC (w/w) SUV. All spectra were taken at room temperature after incubation under the conditions described in Experimental Procedures. The spectrum of the native toxin at pH 7 and 23 °C is shown for comparison (dashed line). The λ_{max} values for the forms of toxin are 329 nm, native (N) state; 330 nm, L′ state; 331 nm, L″ state; 333 nm, R′ state; and 336 nm, R″ state (prepared by heating the R′ state).

emission intensity in the absence of lipid. The transition is largely irreversible (not shown). The decrease in the 330/350 ratio indicates that emission is red shifted (shifted to longer wavelength) in the higher temperature state. This thermally induced red shift suggests a transfer of buried Trp residues to a more polar environment, probably the aqueous environment, and is consistent with thermal unfolding. This latter conclusion is in agreement with the observation of a cooperative endothermic transition at low pH upon heating toxin in solution (Ramsay et al., 1989).

The low-pH conformation has previously been named the L state (Blewitt et al., 1985). Therefore, we call the low-pH form found at lower temperature the L′ state and the form predominating at higher temperature the L″ state. The above results suggest that the more unfolded L″ state, which predominates at 37 °C, is more likely to predominate under physiological conditions in the endosome.

Figure 1 (left) also shows that the thermal transition is observed at about the same temperature in membrane-inserted toxin. (Several previous studies have shown that almost all of the toxin inserts when incubated in the presence of model membranes at low pH (Hu & Holmes, 1984; Zalman & Wisnieski, 1984; Chung & London, 1988).) There is a small overall displacement in the 330/350 curve in the presence of PC vesicles, but this is likely to reflect variablity between samples. The most significant lipid-induced difference is the smaller degree of red shifting of emission during the transition found with PG/PC SUV.[3]

[3] The intensity decrease is also greater without lipid (Chung & London, 1988).

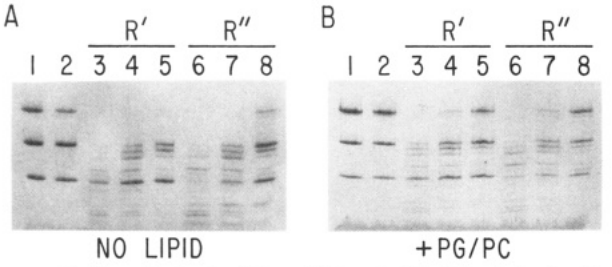

FIGURE 2: Trypsinolysis of low-pH-treated diphtheria toxin after neutralization of pH. The gel in (A) shows digestion in the absence of SUV; (B) shows digestion in the presence of 28% PG/72% PC (w/w) SUV. Samples were incubated at pH 4.5, 23 °C, and then adjusted to pH 7–7.2, 23 °C, to form the R′ state (lanes 3–5) or incubated at pH 4.5, 37 °C, and pH adjusted at 37 °C to form the R″ state (lanes 6–8). Trypsin digestion was carried out at room temperature for 15 min. Other details are given in Experimental Procedures. Lane 1 contains toxin alone. Lane 2 is a control in which digestion was stopped immediately after addition of trypsin (2.5 μg/mL). Trypsin concentration was 2.5 μg/mL in lanes 3 and 6, 0.75 μg/mL in lanes 4 and 7, and 0.25 μg/mL in lanes 5 and 8.

Temperature Dependence of the Conformation of Low-pH-Treated Toxin after pH Neutralization: Fluorescence Measurements. Motivated by the fact that toxin moves from an acidic endosome to the neutral pH cytoplasm, we also examined low-pH-treated toxin after pH neutralization (Figure 1 (right)). At all temperatures, the 330/350 ratio is lower (more red shifted) than that found at pH 4.5, suggesting that after pH neutralization Trp residues are more exposed to the polar aqueous solution (in agreement with fluorescence quenching results described below). As at low pH, after pH neutralization the toxin undergoes a thermal transition upon warming, found at about 35–37 °C (Figure 1 (right)). Also, as at low pH, the transition results in a decrease in overall emission intensity (see spectra in Figure 1 (inset)) and is largely irreversible. The decrease in the 330/350 ratio at higher temperatures shows that the transition involves a further red shift in Trp emission. This (as at low pH) is consistent with a thermal unfolding event resulting in increased exposure of Trp residues to aqueous solution. However, in contrast to the behavior at low pH, the presence of PG-containing vesicles does not strongly affect the amount of red shifting during the thermal transition.

We call the conformations formed after pH neutralization R states (for reversal of low pH), naming the form predominating at lower temperatures the R′ state and the form predominating at higher temperatures the R″ state.

It should be noted that there are two ways to obtain pH-neutralized toxin at high temperature, one by heating pH-neutralized (i.e., R′) toxin and a second by pH neutralizing the toxin preincubated at high temperature (i.e., L″ toxin). The fluorescence of R″ toxin obtained by these two processes is very similar, suggesting that both processes give rise to similar conformations (also see below). If the L″ state is formed under physiological conditions, then one might expect the R″ state to be formed after exposure to the neutral pH of the cytoplasm.

Conformation of Low-pH-Treated Diphtheria Toxin As Determined by Sensitivity to Proteolysis. Because previous studies have shown that trypsin digestion of the toxin is sensitive to conformation (Hu & Holmes, 1984; Dumont & Richards, 1988), the sensitivity of low-pH-treated toxin to proteolysis was determined in order to confirm the fluorescence results. The sensitivity of toxin to proteolysis after pH neutralization of low pH-treated samples is shown in Figure 2. Domain A is clearly more sensitive to proteolysis in the high-temperature R″ state than in the R′ state, while domain B digestion is unchanged by exposure to high temperature.[4] This is true for the R″ state formed by heating the R′ state and for R″ prepared by pH neutralization of the L″ state.

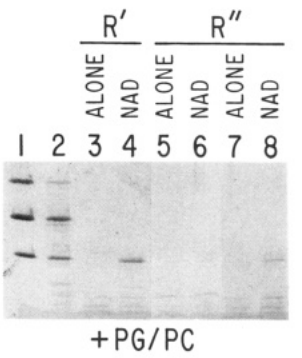

FIGURE 3: Protection of diphtheria toxin in different conformations from trypsinolysis by NAD^+. Samples were prepared as described in Experimental Procedures. Proteolysis was carried out in the presence of 28% PG/72% PC (w/w) with 7.1 μg/mL trypsin for 30 min. Lane 1 contains toxin alone. Lane 2 is the control lane (see Figure 2). Lanes 3 and 4 contain toxin preincubated at pH 4.5, 23 °C, and then adjusted to pH 7–7.2, 23 °C (R′ state). Lanes 5 and 6 contain toxin preincubated as in lanes 3 and 4 except at 37 °C (R″ state). Lanes 7 and 8 contain toxin preincubated as in lanes 3 and 4 and then heated to 45 °C (R″ state). Lanes 3, 5, and 7 are in the absence of NAD^+, and lanes 4, 6, and 8 are in the presence of NAD^+.

Since digestion of the B domain is not affected significantly by the R′ to R″ transition, it is likely that the A domain is undergoing the major conformational change during this transition. Consistent with the fluorescence results, insertion of the toxin into PG-containing vesicles has no effect on the A and B domain digestion pattern.

The change in A domain protection from proteolysis during the L′ to L″ transition at low pH is much less noticeable than in the R′ to R″ transition after pH neutralization, both in solution and when inserted in PC vesicles, and is not seen when inserted in PG-containing vesicles (not shown). These properties may be due to the A domain being more deeply buried at low pH and less exposed to proteolysis in general (see below).

Use of Ligand-Induced Protection from Proteolysis To Assess the Conformation of Toxin after Low-pH Treatment. Binding of NAD^+ and other ligands will protect toxin and isolated domain A in the native state from digestion by trypsin (Kandel et al., 1974; Zhao & London, 1988b). Ligand-induced protection was used to further evaluate the conformation of low-pH-treated toxin. Harsher digestion conditions were used in which there was complete digestion without ligand binding. As shown in Figure 3, protection of the A domain from digestion by NAD^+ was observed in the R′ state but none or very little was seen in the R″ state, whether formed by heating the R′ state or by pH neutralization of the L″ state.[5] Protection was observed both in the absence of lipid (not shown) and when inserted in PG/PC SUV. This suggests that domain A can bind NAD^+ in the R′ state but not in the R″ state and is consistent with the earlier conclusion that the A domain is folded in the R′ state but not in the R″ state.

Measurement of NAD^+ Glycohydrolase Activity To Assess the Conformation of Toxin after Low-pH Treatment. The

[4] The same results were obtained when digestion of toxin exposed to 37 °C was done at 37 °C instead of at 23 °C. We chose to make use of the irreversibility of the thermal conformational changes to do digestions at 23 °C in order to avoid artifacts due to thermal effects on intrinsic trypsin activity.

[5] Use of NAD^+ interactions to assess conformation will not work at low pH due to weaker NAD^+ binding (Papini et al., 1990).

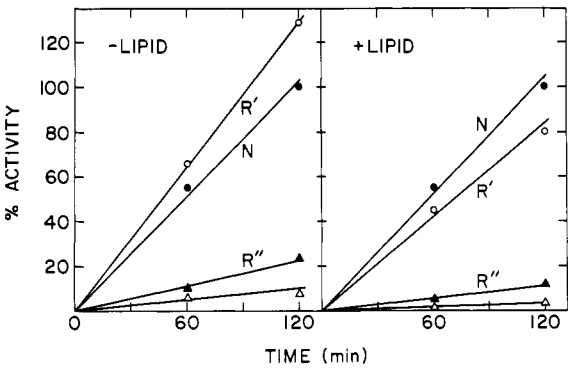

FIGURE 4: NAD^+-glycohydrolase activity of toxin in different conformations assayed in the presence or absence of lipid: left, in the absence of lipid; right, in the presence of SUV composed of 28% PG/72% PC (w/w). Activity was measured in toxin incubated in pH 7 buffer (native state) (●) or after preincubation at pH 4.5, 23 °C, and adjustment to pH 7, 23 °C (R′ state) (○), or after preincubation at pH 4.5, 37 °C, and adjustment to pH 7, 37 °C (R″ state) (▲), or after preincubation at pH 4.5, 23 °C, and adjustment to pH of 7, 23 °C, followed by incubation at 45 °C (R″ state) (△). Activity was measured after samples were brought to 23 °C. For other details of sample preparation see Experimental Procedures.

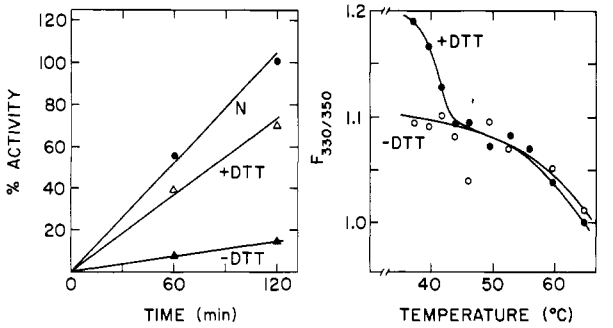

FIGURE 5: NAD^+-glycohydrolase activity and fluorescence of toxin in 28% PG/72% PC (w/w) SUV prepared in the presence or absence of DTT. Samples in the R″ state were prepared by incubation at pH 4.5, 37 °C, followed by adjustment to pH 7–7.2, 37 °C. For other details of sample preparation see Experimental Procedures. The left graph shows the activity of toxin in the native state in the presence of DTT (●) or in the R″ state in the absence of DTT (▲) or in the R″ state after DTT treatment (△). Activity was measured at 37 °C. The right graph shows the effect of increasing temperature on the fluorescence of toxin monitored by the ratio of emission intensity at 330 nm to that at 350 nm in the absence of DTT (○) and in the presence of DTT (●).

proteolysis protection experiments above are complicated by the possibility that the high sensitivity of the R″ state to proteolysis might obscure weak NAD^+ binding. Furthermore, it is possible that some of the ligand binding occurs only after the digestion of the B domain and is not characteristic of A domain behavior in the intact toxin. To overcome these potential problems, we monitored cleavage of NAD^+ by toxin. At neutral pH, the whole toxin and the isolated A fragment of the toxin catalyze the hydrolysis of NAD^+ (and do so at about the same rate (Lory et al., 1980)). This NAD^+-glycohydrolase activity was used to probe the conformation of the A domain of low-pH-treated toxin after pH neutralization (i.e., in the R states). Figure 4 shows that both in the absence of lipid and when membrane-inserted there is full NAD^+-glycohydrolase activity in the R′ state but very little in the R″ state formed by heating the R′ state or by neutralizing the pH of the L″ state.[6] This is consistent with the proteolysis results suggesting that the A domain is folded only in the R′ state.

Effect of Disulfide Reduction upon the Folding and Activity of Membrane-Inserted Toxin after Low-pH Treatment. It has long been known that the isolated A fragment of diphtheria toxin has the remarkable ability to regain activity after incubation under strongly denaturing conditions (Boquet et al., 1976; Pappenheimer, 1977). It has been postulated that if membrane-inserted domain A was unfolded during translocation, it would refold after release into the cytoplasm (Boquet et al., 1976). To determine whether this process can occur, we examined the effect of reducing the disulfide linking the A and B domains upon the degree of folding and activity of the A domain found in the R″ state, which contains partly unfolded and therefore inactive domain A.

As shown in Figure 5 (left), addition of DTT to membrane-inserted toxin in the R″ state reactivates the NAD^+ hydrolyzing activity of the A domain to close to the level observed in native toxin. (In the native state we and previous investigators find that addition of DTT does not affect activity (data not shown; Lory et al., 1980).) Figure 5 (right) shows that at 37 °C there is also a blue shift (towards shorter wavelength) in the 330/350 fluorescence ratio upon DTT addition. This is consistent with the movement of Trp residues to the more nonpolar environment of the protein interior as a result of refolding. If this change does involve refolding, one would expect that unfolding could be observed upon heating the refolded A domain. This is confirmed by the appearance of a cooperative unfolding transition at about 41 °C, which is only observed after DTT addition. Above 41 °C, the 330/350 ratio after DTT addition is the same as before DTT addition when the toxin is in the R″ state, which has an unfolded A domain. In summary, these observations show that reduction of the disulfide link to the B domain does indeed induce the refolding and reactivation of the A domain. It should be noted that we find efficient release of domain A, but not domain B, from model membranes upon addition of DTT (data not shown).

Degree of Diphtheria Toxin Bilayer Penetration in Different Conformations. In order to learn about the depth of membrane penetration of the various conformations of the toxin, we compared the extent of quenching of the Trp fluorescence intensity of membrane-bound toxin by phospholipids carrying spin-labels on either the 5 or 12 carbon atoms along their fatty acid chains (Chattopadhyay & London, 1987).

Table I shows that quenching is strongest for toxin in the L conformations, with slightly greater quenching by the deeper 12-position spin-label relative to the shallower 5-position one, which supports the conclusion that there is deep penetration of toxin into the membrane. This agrees with results obtained by use of lipid photolabeling (Montecucco et al., 1985).

In the R states the degree of quenching is much less than in the L states.[8] This implies that regions of the protein

[6] Similar results were obtained when activity was assayed at 37 °C. There is a small residual activity in the high-temperature-treated toxin at 23 °C, probably due to incomplete unfolding during the high-temperature treatment plus a small amount of refolding upon cooling from a higher temperature.

[7] This assumes the membrane forms a near-normal bilayer around a membrane-embedded toxin molecule. This is consistent with the observation that toxin membrane penetration results from hydrophobic interactions (Hu & Holmes, 1984; Zalman & Wisnieski, 1984; Blewitt et al., 1985; Montecucco et al., 1985) and the agreement of quenching with photolabeling (see text). However, a somewhat distorted bilayer near the protein should not be ruled out.

[8] It is noteworthy that somewhat stronger quenching occurs in the R″ state prepared by pH neutralization of the L″ state than in the R″ state formed by heating of the R′ state. This implies there may be a difference in membrane penetration between the two R″ forms, which are otherwise quite similar.

Table I: Spin-Labeled Phospholipid Fluorescence Quenching of Membrane-Inserted Diphtheria Toxin in Different Conformations[a]

conformation	conditions	F/F_0 for spin-labeled PC: 5 position	F/F_0 for spin-labeled PC: 12 position
L′	pH 4.3, 23 °C	0.60	0.51
L″	prepared by heating L′, 37 °C	0.57	0.52
R′	prepared by pH neutralization of L′, 23 °C	0.88	0.81
R″	prepared by pH neutralization of L″, 37 °C	0.74	0.76
R″	prepared by heating R′, 45 °C	0.92	0.88

[a] F/F_0 is the ratio of toxin fluorescence in the presence of SUV containing 40 mol % spin-labeled PC to that in the presence of SUV with no spin-label. The overall composition of the SUV was 28% PG/72% spin-labeled plus unlabeled PC (mol/mol) in all cases. The different conformations were prepared from the L′ state as described in the inset in Figure 1, with fluorescence measured at the temperatures shown in the table. At pH 4.3, 23 °C, the average apparent depth of the Trp residues calculated by the quenching analysis (Chattopadhyay & London, 1987) is 8.3 Å from the center of the bilayer.[7] Since this value is influenced by the relative intensities of each Trp residue, it is not a direct average.

including several of the Trp residues no longer contact the bilayer, again consistent with photolabeling (Montecucco et al., 1985). However, the Trp remaining within the bilayer is still quenched more strongly by the 12-position quencher than by the 5-position one. This implies that either some portion of the protein or some fraction of the protein molecules remain deeply embedded.[9] The decrease in quenching could alternately be interpreted as suggesting that a large fraction of toxin molecules have dissociated from the bilayer. This does not appear to be the case because centrifugation experiments show that at least most of the toxin remains membrane-associated (Hu & Holmes, 1984; Jiang, J. X., and London, E., unpublished observations).

Discussion

Different Conformations of Membrane-Inserted Diphtheria Toxin. This report has shown that, both at low pH and after pH neutralization, membrane-inserted diphtheria toxin takes on distinct conformations with different degrees of folding. In order to understand translocation of the toxin across membranes, it will now be necessary to characterize each form and the roles they play. The L′ and R′ conformations that dominate at lower temperatures are similar in that they contain folded domain A. The L″ and R″ conformations predominate at higher temperatures and contain domain A that is at least partly unfolded. It should be pointed out that the behavior of toxin after pH neutralization is clearly distinct from the neutral pH behavior of the original hydrophilic native (N) state as shown by differences in fluorescence properties, hydrophobicity, and domain B conformation (Hu & Holmes, 1984; Chung & London, 1988). Figure 6 summarizes the various conformational transformations that the toxin can undergo as based on the data in this report and previous studies (Montecucco et al., 1985; Zhao & London, 1988a).

Since the conformation of domain A in a specific L state is closely related to that in the corresponding R state found at the same temperature, the difference between L and R states must largely involve independent differences in B domain conformation rather than in the A domain. The apparent ability of the A and B domains to undergo independent transitions is supported by the calorimetric studies of Ramsay et al. (1989) and the observations of Papini et al. (1987b) that membrane interaction of the two domains can occur independently.

On the basis of the difference in the depth of insertion of toxin in the L and R states detected by spin-label lipid quenching, it appears likely that the whole toxin, and therefore the B domain, becomes less hydrophobic and less deeply inserted after conversion of the L state to the R state by neutralization of pH. This is supported by observations on B domain isolated under denaturing conditions. We find that after removal of denaturant the degree of isolated domain B insertion into model membranes is much higher at low pH than after pH neutralization as judged by fluorescence quenching (Zhao, J.-M., and London, E., unpublished observations). On the other hand, in whole toxin proteolysis of the B domain is similar in the L and R states (not shown). Since it is much more sensitive to proteolysis in both of these states than in the native state (Hu & Holmes, 1984; Dumont & Richards, 1988), the B domain probably has a somewhat unfolded conformation in both the L and R states than it has in the native state.

Diphtheria Toxin Conformation in the Presence and Absence of Lipid. The results of this study show that after exposure to pH 4.5 the toxin generally takes on similar or identical L and R conformations in the presence and absence of lipid (especially PC SUV). This can be explained by the previous observations that in the absence of lipid the toxin is highly aggregated at low pH (Blewitt et al., 1985; Collins & Collier, 1987). Since the toxin is very hydrophobic at low pH, it is very likely that there are strong hydrophobic interactions between toxins inducing this aggregation (Blewitt et al., 1985; Collins & Collier, 1987). In the absence of lipid, contacts between the hydrophobic sites on adjacent toxin molecules in these aggregates may effectively substitute for the contact of hydrophobic sites on the membrane-inserted toxin with lipid. This would allow formation of a conformation in the absence of lipid that is similar to the conformation in the membrane-inserted state.[10]

The most marked lipid effects on toxin behavior are seen with negatively charged PG-containing SUV at low pH. On the basis of our previous studies, it is likely that electrostatic interactions somehow modify toxin behavior in the presence of PG (Chung & London, 1988; Zhao & London, 1988a). The fact that a PG effect is seen at low pH but not after pH neutralization is consistent with the behavior of isolated fragment A (Zhao & London, 1988a) and suggests that PG influences A domain behavior in whole toxin. We cannot be specific about the nature of the change induced by the inclusion of PG at this point. It should be noted that Farahbakhsh and Wisnieski (1990) have also noticed lipid effects on the structure of acid-treated toxin.

Role of the Different Conformations of Membrane-Inserted Toxin in Translocation. We also found that by treatment of

[9] Another possibility is that the toxin aggregates within the plane of the membrane upon pH neutralization, thereby excluding lipid molecules and decreasing quenching. However, our preliminary results with self-quenching by rhodamine-labeled toxin indicate aggregation does not increase after pH neutralization (not shown).

[10] Interestingly, upon neutralization of toxin preincubated at pH 3, rather than 4.5, there is a difference in toxin behavior in the absence and presence of lipid. In the presence of lipid we find behavior is the same as after pH 4.5, but in the absence of lipid most toxin molecules return to the native state after pH neutralization. This is demonstrated by markedly decreased sensitivity of domain B to trypsin, protection of toxin from trypsin digestion by A_pU_p (which only binds tightly to native toxin (Collins & Collier, 1987)), and previous studies of activity and conformation (Sandvig & Olsnes, 1981; Blewitt et al., 1985). The probable reason for the difference in pH 3 and pH 4.5 reversibility is the lesser aggregation of the toxin at pH 3 (Blewitt et al., 1985; Collins & Collier, 1987).

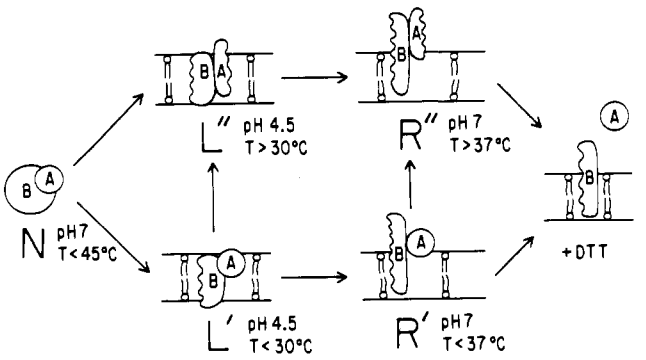

FIGURE 6: Illustration of the different conformations of diphtheria toxin described in this report. We have not shown that R″ formed from R′ and L″ may not be identical, that L conformation may not be the same in PC and PG/PC vesicles, and that the B domain may partly surround the A domain.

pH-neutralized toxin with a reducing agent the A domain refolds upon the cleavage of the link to the B domain and release from the membrane-bound state. This is consistent with translocation models that predict that refolding of domain A occurs after the translocation into the cytoplasm. In addition, it has been previously demonstrated that upon partial unfolding at low pH isolated domain A becomes hydrophobic and gains the ability to insert into model membranes (Zhao & London, 1988a). It also appears that the conformations that contain unfolded domain A, L″ and R″, should predominate at 37 °C. Together, these observations suggest that the L″ and R″ forms are involved in translocation in vivo. However, further study will be needed because in vivo the equilibrium between low- and high-temperature forms may be influenced by factors absent in our model system, such as binding to a receptor protein, interactions with specific lipids, exact pH, absence of a transmembrane pH gradient (Kagan et al., 1981; Sandvig et al., 1988), the effect of nicking between A and B domains, and C-terminal proteolytic processing[11] (Hu & Holmes, 1987; Moskaug et al., 1989).

Measurement of the degree of translocation could in principle distinguish which conformations are able to undergo translocation. In preliminary studies, we find that efficient translocation can be obtained by measuring the release of domain A from model membrane encapsulated toxin at both 23 and 37 °C (unpublished observations). However, the possibility of translocation arising from nonspecific leakiness requires further studies before such experiments can be interpreted unambiguously. In addition, it should be possible to gain vital clues to the translocation mechanism by determining in which conformation(s) the membrane-associated domain A has crossed the bilayer. Characterizing translocation by these methods will be among the goals of our future work.

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[11] Variable C-terminal processing can affect membrane insertion and translocation and may explain why we found the temperature of the L′ to L″ transition in one of our preparations occurred a few degrees higher than in this report.

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Molecular Dynamics Simulations of the Unfolding of an α-Helical Analogue of Ribonuclease A S-Peptide in Water†

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ABSTRACT: Molecular dynamics simulations of the S-peptide analogue AETAAAKFLREHMDS have been conducted in aqueous solution for 300 ps at 278 K and for 500 ps in two different runs at 358 K. The results show agreement with experimental observations in that at low temperature, 5 °C, the helix is stable, while unfolding is observed at 85 °C. In the low-temperature simulation a solvent-separated ion pair was formed between Glu-2 and Arg-10, and the side chain of His-12 reoriented toward the C-terminal end of the α-helix. Detailed analyses of the unfolding pathways at high temperature have also revealed that the formation or disappearance of main-chain helical hydrogen bonds occurs frequently through an $\alpha \rightleftharpoons 3_{10} \rightleftharpoons$ no hydrogen bond sequence.

It is well known that proteins display a widely varied range of distinct three-dimensional structures and topologies based on a very limited "alphabet" of only twenty L-amino acids (Richardson, 1981), albeit in am almost infinite number of possible combinations. Given that the spatial arrangement of the atoms of a given protein determines most of its properties, including catalytic power and specificity, correlating the residue sequence to the three-dimensional structure has been a long sought goal. In spite of the apparent enormity of the problem, a large stimulus was provided by the classical experiments of Anfinsen on the unfolding and refolding of ribonuclease A in vitro (White & Anfinsen, 1959). These experiments showed that the amino acid sequence of ribonuclease A contains enough information to provide folding into the "correct" (i.e., native) conformation. This phenomenon has been observed subsequently for other small single-domain proteins (Baldwin, 1989). The many attempts to formulate models that explain how a given amino acid sequence folds into the specific three-dimensional structure of the corresponding protein have given rise to the so-called "protein-folding" problem (King, 1989).

Previous investigations on the folding pathways of proteins have lead to the development of two general classes of models, kinetic and structural. The kinetic models have been derived

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