Involvement of Denaturation-like Changes in Pseudomonas Exotoxin A Hydrophobicity and Membrane Penetration Determined by Characterization of pH and Thermal Transitions

ROLES OF TWO DISTINCT CONFORMATIONALLY ALTERED STATES*

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Previous investigators have shown that exotoxin A undergoes a conformational switch to a hydrophobic state at low pH. This change appears to play a role in exotoxin A entry into cells by facilitating its penetration of the membranes of acidic organelles. We have examined the effects of pH, temperature, and denaturants in order to define the role of conformational changes in membrane penetration by the exotoxin. We find that two distinct low pH conformations exist. An intermediate low pH state (L1) dominates at pH 3.7-5.4 and is distinguished by blue-shifted fluorescence and weak or no hydrophobicity. The second low pH state (L2) is dominant below pH 3.7 and is characterized by red shifted fluorescence and strong hydrophobicity. L1 is a folded state as judged by its spectroscopic properties and the observation that it undergoes distinct and cooperative thermal and denaturant induced unfolding transitions. L2 appears to be more like a denatured state, as it shows no cooperative thermal or denaturant induced transitions and has spectroscopic properties very similar to exotoxin A that has been thermally denatured at pH 7. Exotoxin A in the L1 state strongly binds detergent micelles and binds and inserts into model membranes. Therefore, denaturation-like conformational changes appear to play an important role in membrane insertion. The pH of the transition to a membrane-inserting state is influenced by the composition of the model membranes and is close to pH 5 in the presence of vesicles containing a phosphatidylglycerol/phosphatidylcholine mixture. These vesicles probably promote formation of the L1 state via mass action effects. The implications of these results for membrane penetration and translocation of proteins without apparent hydrophobic regions, such as exotoxin A, is discussed.

Pseudomonas exotoxin A is a 67-kDa toxin protein secreted by Pseudomonas aeruginosa. Like diphtheria toxin, exotoxin A is translocated across membranes of sensitive eukaryotic cells and inhibits protein synthesis by catalyzing the transfer of ADP-ribosyl moiety of NAD+ onto elongation factor 2 (1, 2). The sequence and three-dimensional structure of exotoxin A indicate that the molecule has three structural domains (3-6). There is significant sequence homology between diphtheria toxin and exotoxin A in their respective catalytic domains but little if any in the remainder of the molecules (7-12).

Understanding exotoxin A action and how it enters cells are of interest because of the effort to develop improved immunotoxins for therapeutic applications. Exotoxin A has been used extensively in the design of immunotoxins, especially by Pastan and co-workers (13, 14). In addition, determination of the mechanism of membrane translocation of exotoxin A and related toxins should have important implications for the regular membrane translocation of cellular proteins and entry of viruses into cells.

Present evidence indicates that exotoxin A enters cells by receptor-mediated endocytosis (15). Subsequent exposure to the low pH in the lumen of acidic organelles is a critical step in the translocation of exotoxin A into the cytoplasm (16, 17). Studies with isolated exotoxin A have shown that low pH is sufficient to induce its insertion into model membranes (see below). In these respects, entry of exotoxin A is very similar to that of diphtheria toxin. Nevertheless, the exact site of exotoxin A membrane penetration and whether additional processing of the exotoxin or interactions with other proteins influence translocation are not yet certain (15).

In order to clarify the membrane translocation step, the effect of low pH upon the properties of the isolated exotoxin has been studied by Wisnieski and colleagues (18-21). They have shown that as in the case of diphtheria toxin (22), low pH induces a dramatic conformational switch from a hydrophilic to a hydrophobic membrane penetrating conformation. In this report we extend these studies to demonstrate that exotoxin A has two distinct low pH conformations with very different properties and characterize these conformational changes. The results give a new picture of the various exotoxin A conformations and their role in membrane penetration. Comparison with the low pH behavior we have previously characterized for diphtheria toxin (22, 24, 26, 27) shows significant similarities and differences between the two toxins. As in the case of diphtheria toxin, a low pH-induced denaturation-like event is linked to induction of the membrane penetrating state. These results have important implications for the translocation of the exotoxin across membranes. They also allow us to suggest how proteins without apparent hydrophobic regions become hydrophobic and insert into membranes.

EXPERIMENTAL PROCEDURES

Materials—Pseudomonas exotoxin A was purchased from List Biochemicals. The commercial toxin gave a single band upon SDS1 gel

1 The abbreviations used are SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicles.
electrophoresis and had the same cytotoxicity to Vero cells as reported previously (23). Brj 96 was purchased from Sigma. Brominated Brj 96 was prepared as reported previously (24, 25). Bromination was most stable after pH neutralization when freshly prepared saturated Brj/H,0 was used. Dioleoyl phosphatidylglycerol, egg phosphatidylcholine, and brominated phosphatidylcholine (1,2-di-(9,10-dibromo) stearoyl-sn-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids (Pelham, AL). Lipid purity and concentration were confirmed by thin layer chromatography and gravimetric analysis as described previously (26).

Sample Preparation—Small unilamellar vesicles (SUV) were prepared by sonication. Mixtures of the appropriate lipids in CHCl3 were first dried under a stream of N2 and then further dried under high vacuum for at least 1 h. The dried lipids were suspended in 10 mM NaCl at a concentration of 10 mg/ml, deoxygenated with N2, and then sonicated in an argon-flushed tube for 30–60 min using 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.

Unless otherwise noted, samples contained a final concentration of 10 mM buffer with or without 150 mM NaCl and with or without detergent or SUV. In the final step, 1/10 of 1 mg/ml exotoxin A dissolved in 10 mM sodium phosphate, 150 mM NaCl, pH 7, was added to give a final volume of 1 ml. The buffers used were sodium formate below pH 4, acetate between pH 4 and 6, and phosphate at pH 6 and 7. Spectroscopic measurements were performed at room temperature after incubation of the samples for at least 30 min at room temperature (about 23°C).

Fluorescence Spectroscopy—Fluorescence was measured with a Spex 212 Fluorolog spectrofluorimeter. Semimicro quartz cuvettes with a 10-mm internal length and a 4-mm internal width were used. Slit widths were 1.25 mm (band pass 2.25 nm) for excitation and 5 mm (band pass 9 nm) for emission. Fluorescence intensity was generally measured at 280-nm excitation and 330-nm emission. Background intensity in samples without protein was subtracted. In acrylamide quenching experiments, excitation was set at 295 nm and emission 340 nm. Each sample was titrated with a small aliquot of 5 M acrylamide, and fluorescence values were corrected for dilution. Inner filter effects due to acrylamide were only a few percent at the highest concentrations used and were not corrected. The temperature dependence of fluorescence was determined as described previously (27). Buffers that have a negligible dependence of pH upon temperature were used (27).

Gel Electrophoresis and Differential Scanning Calorimetry—SDS gel electrophoresis was done using a Phastsystem (Pharmacia LKB Biotechnology Inc.). 10–15% gradient gels were used and protein bands visualized by Coomassie Blue staining. The Phastsystem was also used to determine the isoelectric point of exotoxin A at 15 °C. Samples of 0.5 μg of protein dissolved in 1 ml of water were applied both at the loading positions closest to the anode and closest to the cathode on a native pH 4–6.5 gradient gel. Electrophoresis was performed according to manufacturer's directions. Exotoxin A migrated to the same central point independent of loading position confirming that it reached its true isoelectric point at the end of the electrophoresis period. pl was determined by comparison with standard proteins (Sigma) electrophoresed following the same loading procedures on the same gel (soybean trypsin inhibitor, pl = 4.55; β-lactoglobulin, pl = 5.13; carbonic anhydrase B, pl = 5.85). Calorimetric scans were performed on a Microcal MC2 differential scanning calorimeter interfaced to an IBM-PC computer. Samples contained 0.3–0.35 mg/ml exotoxin A in the same buffers used for fluorescence measurements. Temperature was increased at a rate of 60 °C/h.

RESULTS

The Effect of pH and Other Agents upon Exotoxin A Fluorescence—The effect of pH upon the intrinsic Trp fluorescence of the exotoxin is shown in Fig. 1. In the presence of 150 mM NaCl, fluorescence intensity detects two conformational changes, one occurring at about pH 5.4 and the other at pH 3.7. We call the state predominating at neutral pH to pH 5.4, the native (N) state; the intermediate state predominating between pH 5.4 and 3.7, the first low pH state (L1); and the state predominating below pH 3.7, the second low pH state (L2). The transitions between these states can also be detected by a shift in the wavelength of maximum emission, which we have monitored by the ratio of emission intensity at 330 nm to that at 350 nm (see text). Inset, emission spectra at pH 7 (curve A); pH 4.6 (curve B); pH 3.0 (curve C); pH 7 and 60 °C (curve D). Bottom, intensity at 330 nm. Samples contained 10 μg/ml exotoxin A in 10 mM buffer and 150 mM NaCl (O) or no NaCl (O). See “Experimental Procedures” for details.

FIG. 1. Effect of pH upon the fluorescence of exotoxin A. Top, emission maximum shifts as monitored by the ratio of intensity at 330 nm to that at 350 nm (see text). Inset, emission spectra at pH 7 (curve A); pH 4.6 (curve B); pH 3.0 (curve C); pH 7 and 60 °C (curve D). Bottom, intensity at 330 nm. Samples contained 10 μg/ml exotoxin A in 10 mM buffer and 150 mM NaCl (O) or no NaCl (O). See “Experimental Procedures” for details.

Decreasing basic strength by leaving out NaCl shifts the pH of the transitions in different directions. The L1 to N midpoint shifting up about 0.1–0.2 units as well as becoming more gradual and the L2 and L1 transition shifting down about 0.5 units (Fig. 1). This result probably explains the conflict between the data of two previous studies on the effect of NaCl on the low pH-induced changes in exotoxin A. One study evidently examined NaCl effects on the N to L1 transition and the other on the L2 to L1 transition (20, 29). We found no effect on the transition pH upon inclusion of 0.5 mM MgCl2 plus 0.5 mM CaCl2 or upon inclusion of 10 mM EDTA (data not shown). After preincubation of the exotoxin for 1 h in 50 mM dithiothreitol to reduce accessible disulfide bonds, only one pH transition near 5.5 was detected.

Acrylamide quenching of fluorescence was used to evaluate the effect of pH upon exposure of Trp residues to solution. Higher exposure of Trp residues give rise to stronger quenching which results in a higher slope of a plot of Fc/F versus acrylamide concentration (22). Fig. 2 shows the apparent exposure of Trp is least in the N state, higher in the L1 state, and highest in the L2 state. It should be cautioned that since several Trp residues are involved and since quenching is affected by the fluorescence lifetime of the Trp residues, exposure cannot be evaluated very quantitatively at present (see “Discussion”).

Effect of pH upon Proteolysis—Proteolysis was chosen as a second method to investigate pH-induced conformational changes. Previous work had shown increased digestion by
Exotoxin A Conformation

FIG. 2. Acrylamide quenching of exotoxin A fluorescence. Samples contained 10 μg/ml exotoxin A in 10 mM buffer plus 150 mM NaCl. ○, pH 7.0; △, pH 3.8; ●, pH 4.5.

FIG. 3. Effect of pH upon the proteolysis of exotoxin A by trypsin (left) or papain (right). Samples containing 250 μg/ml exotoxin A in 4 μl of solution containing 10 mM buffer, 150 mM NaCl, and 25 μg/ml trypsin or 25 μg/ml papain were incubated for 30 min at room temperature. The pH of the buffer is shown above the appropriate lane. The reactions were stopped by the addition of 1 μl of 1 mg/ml soybean trypsin inhibitor or 1.5 μl of 10 mg/ml HgCl₂, respectively. No digestion was found in controls at pH 7 and 4.5 for trypsin and pH 3.3 for papain in which the 30-min incubation was omitted. The upper and lower × symbols indicate bands due to trypsin and soybean trypsin inhibitor, respectively.

trypsin at pH 4 relative to 7 (20). Fig. 3 shows the effect of pH upon proteolysis by trypsin and papain. Trypsin detects the N to L₄ transition and indicates that additional sites become exposed in the L₄ state. Papain detects the L₁ to L₄ transition and shows an additional site or sites become(s) exposed in the L₄ state. These results are in good agreement with those observed by fluorescence. The apparent transition pH determined from proteolysis can be slightly shifted from that determined by fluorescence due to the perturbation of conformational equilibria by protease action. As a low pH state is digested away, equilibrium will shift additional molecules into the low pH form, allowing extra digestion.

Effect of Temperature and Denaturants on Exotoxin A Conformation—In order to define the conformation of exotoxin A at different pH values more precisely, the effect of temperature upon the fluorescence of the three different pH-dependent conformational states was examined, as shown in Fig. 4.

A clear thermal denaturation is observed with a midpoint at 56 °C for the native conformation at pH 7, and at 48 °C for the L₄N conformation at pH 4.6. No transition is observed for the L₄N at pH 3. In fact the λₘₐₓ, of emission is almost the same for the L₄N conformation and the thermally-denatured conformation at pH 7 (Fig. 4, inset). The results of differential scanning calorimetry confirm those of fluorescence. Endothermic transitions having similar ΔH values are observed at pH 7 and pH 4.6 at the same temperatures at which fluorescence changes occur. As in fluorescence, no calorimetric transition can be detected at pH 3 (data not shown). Together, these results demonstrate that the native and L₄N conformations contain folded regions at room temperature, whereas the L₄N conformation behaves like a denatured state.

By measuring the thermal dependence of fluorescence at additional pH values, the predominating conformation at any pH-temperature conformation was determined. The results are summarized in the phase diagram shown in Fig. 5. With the exception of the existence of the L₁ state in exotoxin A, the overall pH-thermal behavior of exotoxin A is similar to that of diphtheria toxin (27), especially if the L₄N state is equated with the hydrophobic diphtheria toxin L state (see "Discussion"). It is noteworthy that heating the L₄N state at pH 5 seems to result in a transition to the native state at higher temperatures.

Additional details were obtained by examining the effects of denaturants upon conformation at different pH values. Cooperative unfolding transitions are observed at pH 7 and pH 4.6 as denaturant concentration is increased (Fig. 6). At pH 7 the concentration of denaturant at the midpoint of the unfolding transition is 3.8 M urea or 1 M guanidinium Cl.

At pH 4.6 the midpoint concentration is only slightly less, 3.6 M urea or 1 M guanidinium Cl. In contrast to these results, no cooperative transition is observed at pH 2.9, consistent with the idea that the L₄N conformation is in a denatured-like state. Instead, at pH 2.9 additional unfolding gradually occurs when denaturant concentration is increased, as detected by the gradual decrease in fluorescence intensity shown and a large gradual red shift in the emission maximum (not shown). This shows that the L₄N state in the absence of denaturants is not...
Membrane Interactions—To correlate the changes in conforming groups. In these assays, association is measured by the fluorescence quenching method we applied previously to diphtheria toxin (22, 24-26). This method detects association of exotoxin A hydrophobicity and insertion into SUV model membranes was examined. To assay these interactions, we used the fluorescence quenching method we applied previously to diphtheria toxin (22, 24-26). This result is most easily explained in terms of a mass action effect due to electrostatic interactions between lipid and exotoxin A, which was the same as the totally unfolded random coil state found at high concentrations of denaturants. This result is also very similar to that found for the L state of diphtheria toxin (27).

The Effect of pH on Exotoxin A Hydrophobicity and Model Membrane Interactions—To correlate the changes in conformation with membrane interactions, the effect of pH upon exotoxin A hydrophobicity and insertion into SUV model membranes was examined. To assay these interactions, we used the fluorescence quenching method we applied previously to diphtheria toxin (22, 24-26). This method detects association by the quenching of Trp fluorescence when protein binds to micelles or model membranes containing attached quenching groups. In these assays, association is measured by the reduction of fluorescence intensity in the presence of micelles or membranes containing brominated quenchers (F) relative to the intensity in the presence of micelles or membranes without quenchers (F₀). Binding is indicated by a F/F₀ ratio less than one and a lack of binding by a F/F₀ ratio equal to 1. It should be noted that because quenching of bound protein is never complete, the fraction of quenching (1 - F/F₀) is only the lower limit to the fraction of protein bound.

The binding of exotoxin A to Brij 96 detergent micelles is shown in Fig. 7 (right). Binding to micelles is a standard indicator of hydrophobicity (32). Hydrophobicity appears at about the pH of the L to Lᵢ transition, indicating the Lᵢ state is the hydrophobic state. Omitting NaCl decreases the hydrophobicity transition by about 0.5 pH units. This decrease is the same as observed in the Lᵢ to Lᵢᵢ conformational transition when NaCl is omitted (see above), which further supports the idea that the Lᵢ state is the hydrophobic state. These results also agree with that obtained with the Triton X-114 phase partition assay and the response of the low pH transition of diphtheria toxin to NaCl (24, 29).

The quenching method was also used to measure the pH dependence of exotoxin A binding to model membranes. The results show that the exotoxin binds to model membranes at low pH (Fig. 7, center, left). With phosphatidylcholine SUV, the pH of the transition to the lipid binding state is near the Lᵢ to Lᵢᵢ conformational transition, indicating that the Lᵢᵢ state binds to the membranes. The strong quenching also implies that the exotoxin A is inserting deeply into the model membranes, because the bromine atoms that induce quenching are attached to the 9 and 10 carbons of the fatty acyl chains in brominated phosphatidylcholine, and quenching by bromine is a very short range process (33, 34). These results are consistent with previous experiments that have shown membrane insertion at low pH using photolabeling agents (18, 19).

Omitting NaCl decreased the binding/insertion transition pH by about 0.5 units (Fig. 7, left). This agrees with the shift observed with detergent and is again expected if the Lᵢᵢ conformation is the lipid binding state. Inclusion of 17% of the anionic lipid phosphatidylglycerol caused an increase in the transition pH by about 0.8 units (Fig. 7, center, left). With phosphatidylcholine SUV, the pH of the transition to the lipid binding state is near the Lᵢ to Lᵢᵢ conformational transition, indicating that the Lᵢᵢ state binds to the membranes. The strong quenching also implies that the exotoxin A is inserting deeply into the model membranes, because the bromine atoms that induce quenching are attached to the 9 and 10 carbons of the fatty acyl chains in brominated phosphatidylcholine, and quenching by bromine is a very short range process (33, 34). These results are consistent with previous experiments that have shown membrane insertion at low pH using photolabeling agents (18, 19).

In addition, as in the case of diphtheria toxin fragment A, there may also be an effect on transition pH because of a small change in vesicle size upon inclusion of dioleoyl phosphatidylglycerol (26).
we find to have an isoelectric point of 5.0 (see “Discussion” and “Experimental Procedures”).

**DISCUSSION**

The Two Low pH States and the Role of Denaturation-like Conformational Changes—Farahbakhsh and Wisnieski (21) very recently noted that exotoxin seemed to be less hydrophobic at pH 5 than at pH 4 and briefly speculated that exotoxin had different conformations at these different low pH values. The results of this study demonstrate that exotoxin A does indeed take on two very distinct conformations at low pH.

In the Ln (intermediate) state the protein behaves like a folded protein, as judged by its ability to undergo highly cooperative denaturation transitions. As the transition temperature, enthalpy change, and denaturant concentration in the denaturation process are similar for the native and Ln states, it is likely that the protein remains largely folded in the Ln state. Nevertheless, other data suggest that some region has denatured in the Ln state. Proteolysis data show some buried sites become more exposed in the Ln state, consistent with partial denaturation. Also, we find that the Ln state seems to aggregate in solution and aggregation often accompanies denaturation. The changes in Trp exposure in the Ln state are complex, but overall suggest increased exposure of buried sites. Changes in acrylamide quenching and fluorescence intensity (20) are consistent with more Trp exposure in the Ln state than in the native state. However, the λmax values are consistent with Trp, being more buried in the Ln state than the native state.

The behavior of the Ln state is very different from that of the Ln state. However, Ln behavior is clearly very similar to that of the membrane-penetrating low pH (L) conformation found in diphtheria toxin. This relationship involves many properties including similar increases in Trp exposure relative to the native state, increased susceptibility to proteolysis relative to native states, absence of cooperative unfolding transitions, similarity between the low pH states and the thermally denatured conformations at neutral pH, hydrophobicity relative to native states, membrane penetration, and effects of NaCl concentration on stability (22, 24, 27). These properties are consistent with the Ln state being like a denatured state, as is the diphtheria toxin L state (22, 27), although, as in the case of the Ln state (27), the Ln state is not a random coil state (see “Results”). It is possible that the Ln state and the diphtheria toxin L state correspond to the so-called molten globule denatured state, a compact nonrandom coil denatured state with disordered tertiary structure recently identified in a number of proteins (35–39).

Hydrophobicity, Membrane Insertion, and the Origin of Hydrophobic Behavior in Proteins without Hydrophobic Sequences—The behavior of the exotoxin raises the question of how a protein without apparent hydrophobic sequences, like the exotoxin (4), is able to bind to detergents and penetrate into membranes. As in the case of diphtheria toxin fragment A (20, 40), several factors are probably involved. First, there is the direct increase in hydrophobicity due to protonation and the resulting loss of charge at Asp and Glu residues. Second, the partial unfolding of the protein should result in exposure of additional hydrophobic residues, perhaps in the form of a folded hydrophobic core made up of residues from different parts of the primary sequence. Third, favorable electrostatic interactions between negatively charged lipid and the membrane-inserting conformation can also induce hydrophobicity.

A contribution of electrostatic interactions to model membrane binding and insertion of exotoxin A have been shown by previous studies that find that exotoxin A binds tighter to (and inserts more in) vesicles containing negatively charged lipids compared with vesicles containing only zwitterionic lipids (19). The observation in this report that there is an increase in the transition pH for insertion from pH 4 in the presence of zwitterionic vesicles to pH 5 upon inclusion of negative lipid in the vesicles is also consistent with this conclusion because it indicates that the tight binding in the pH 4–5 range only occurs in the presence of negatively charged vesicles.6 These results are also consistent with the fact that exotoxin A is positively charged in this pH range as shown by its pi (see “Results”). Finally, similar electrostatic contributions to membrane insertion have been observed previously for diphtheria toxin, its A fragment, and proteins containing a signal sequence targeting them to mitochondria (27, 40–42).

Overall, the interaction between exotoxin A and negatively charged vesicles shows striking parallels to that between any protein and SDS. Like negative lipid-exotoxin A interactions, SDS-protein interactions involve a denaturation process in which protein takes on a nonrandom coil state (43). In addition, in both cases both hydrophobic and electrostatic components contribute to the interactions (43). Furthermore, in both cases the hydrophobic interactions do not require that the protein has distinct easily identifiable hydrophobic sequences. Therefore, for some membrane-inserting proteins and peptides the underlying processes involved in SDS-protein interaction and negative lipid-protein interaction may be more similar than realized previously.

Another question is which exotoxin A conformation(s) can insert into model membrane vesicles. The pH dependence of exotoxin A binding to micelles and phosphatidylcholine membranes and the effects of NaCl show that the Ln state is the hydrophobic state. The binding to negatively charged vesicles up to pH 5 can be adequately explained by a lipid-induced shift in the L1-L2 equilibrium toward the L2 state resulting from stronger binding of negative lipid to protein.6 It is also possible that the L1 state has the ability to insert into membranes containing negative lipids. However, one would expect the L1 state to be hydrophobic in order to be able to insert and there is no evidence for it being strongly hydrophobic (see “Results”).

**Implications for Translocation and Further Comparison of Exotoxin A and Diphtheria Toxin Behavior**—Because the exotoxin can penetrate membranes containing a physiologically reasonable fraction of anionic lipids at pH 5, it is certainly conceivable that low pH can directly trigger exotoxin A penetration of endosomes. On the other hand, the physiological function of low pH may be to induce formation of the Ln state, which may be affected by other factors to give rise to insertion. In this regard, it is interesting that the exotoxin is like the A fragment of diphtheria toxin in that they both lack apparent hydrophobic regions and yet can become hydrophobic at low pH. The similarity of the exotoxin to diphtheria toxin fragment A is even closer, because the pH at which fragment A becomes hydrophobic is about the same as the pH at which exotoxin A becomes hydrophobic.

6 In other words, mass action is involved. If a lipid binds to the hydrophobic form of a protein, but not to the hydrophilic form, then the tighter the binding of lipid and the more the equilibrium shifts to the hydrophobic state. If low pH also controls the equilibrium between hydrophilic and hydrophobic states, favoring the latter, then a cumulative mass action effect results in a higher transition pH in the presence of negative lipid (26). Smaller mass action effects would explain the smaller increase in the transition pH for exotoxin A in the presence of phosphatidylcholine relative to that in solution with out any lipids.

that for the exotoxin and shows the same pronounced sensitivity to the presence of negatively charged lipid (27, 40). Since diphtheria toxin fragment A appears to need diphtheria toxin fragment B to complete translocation, it is reasonable to wonder whether some additional factors are needed for the exotoxin as well.

Another interesting comparison between the exotoxin and diphtheria toxin involves the difference in the relative usage of Lys and Arg residues at basic residues. As we have pointed out previously, the catalytic domains of the exotoxin and several other toxins show a strong preference for the use of Arg relative to Lys, whereas in the case of diphtheria toxin catalytic domain there is a bias toward Lys use relative to Arg (44, 45). Because Arg residues are more difficult to deprotonate due to their higher $p_K_a$ and in some cases seem to bond more strongly than Lys residues (45-47), it is conceivable that this sequence bias helps regulate the tendency of particular exotoxin and diphtheria toxin domains to undergo denaturation-like changes at low pH. Future characterization of the behavior of the individual domains of these toxins should yield additional insights into the mechanism of toxin translocation across membranes.

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