Membrane Translocation of Charged Residues at the Tips of Hydrophobic Helices in the T Domain of Diphtheria Toxin†

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ABSTRACT: The low pH triggered membrane insertion of the T domain of diphtheria toxin is a critical step in the translocation of the C domain of the toxin across membranes in vivo. We previously established that the T domain can interact with membranes in two distinct conformations, one in which the TH8/TH9 helical hairpin lies close to the bilayer surface and a second in which it inserts more deeply and appears to be transmembraneous. The loss of charge on residues E349 and D352 due to protonation at low pH has been proposed to be a critical step in transmembrane insertion, because they are within a loop connecting TH8 and TH9, and must cross the membrane upon transmembrane insertion. In this report, the role of these residues was examined by measuring the effect of the double substitution E349K/D352K on the conformation of the TH8/TH9 hairpin through a fluorescent group attached to TH9. At pH 4.5, there was shallower insertion of TH8/TH9 of the E349K/D352K mutant relative to T domain with wild-type residues at 349 and 352. In addition, smaller and/or fewer pores were obtained with the E349K/D352K mutant relative to the wild-type. On the other hand, high T domain concentrations, or further decreasing pH, allowed transmembrane insertion of both the wild-type and the 349K/352K mutant as well as induction of larger and/or more numerous pores. Furthermore, the transmembrane insertion process was rapid for both the mutant and wild-type. This shows that the mutant has the capacity to form a transmembrane structure similar to that of the wild-type T domain and, thus, that introduction of charged groups in membrane-penetrating regions of a protein does not introduce an insurmountable barrier to transmembrane movement. The linkage between the ability of the T domain to form the transmembrane conformation and pores suggests that the effects of these mutations in inhibiting pore formation are likely to partly result from the inability to insert properly. Additionally, the observation that decreasing pH allows the 349K/352K mutant to insert deeply indicates that there are residues other than E349 and D352 whose protonation promotes transmembrane insertion.

Diphtheria toxin is a protein secreted by *Corynebacterium diphtheriae*. It can be split by proteolysis into two chains, A (M_r 21 kDa) and B (M_r 37 kDa), joined by a disulfide bond (*1*). The crystal structure of the toxin shows it consists of three domains (*2*−*5*). The A chain is the catalytic (C) domain. The B chain contains the transmembrane (T) and receptor binding (R) domains. Membrane penetration has been proposed to occur after receptor-mediated endocytosis (*6*). The low pH within the endosomal lumen induces partial unfolding of the toxin which results in exposure of hydrophobic regions and translocation of the A chain into the cytoplasm (*6*). Once in the cytoplasm, the A chain catalyzes the transfer of ADP-ribosyl group of NAD^+ to elongation factor 2, inactivating protein synthesis.

The T domain is made up of a number of α-helices, several of which contain hydrophobic sequences that play a critical role in membrane insertion and translocation (*2*−*7*). Several recent studies have concentrated on two of the most hydrophobic helices, TH8 and TH9, as they are the most likely to form a transmembrane structure upon insertion into a bilayer (*8*−*15*). The protonation of anionic residues on these and other helices that may enter the bilayer is believed to play an important role in regulation of transmembrane insertion (*2*, *16*−*18*). Protonation eliminates the charge on such residues, which should make transmembrane insertion both more rapid and efficient (*2*, *6*, *16*).

Several studies have concentrated on the role of residues E349 and D352 in the insertion process. These residues are in loop TL5, which bridges hydrophobic helices TH8 and TH9, and must pass through the membrane and reach the trans side of the bilayer (i.e., the side opposite that from which insertion occurs) upon transmembrane insertion of TH8 and TH9. For this reason, it was predicted that the protonation of these residues, together with the low pH-induced exposure of TH8 and TH9 to solution, would be especially important for transmembrane insertion (*2*, *16*). This model for triggering of transmembrane insertion by "helical tip neutralization" has been supported by studies demonstrating that reversing the charge on residues E349 and D352 by replacement with Lys decreases toxicity, decreases pore formation and decreases A chain translocation into the cellular cytoplasm (*16*, *18*). These mutations do not seem to affect the low pH-induced conformational transition

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to a hydrophobic state in solution (*16*), but whether they prevent transmembrane insertion or simply result in an altered transmembrane conformation unable to carry out A chain translocation has not been determined (*19*).

In this report, the effect of the double charge reversal mutation, E349K/D352K, on transmembrane insertion of the T domain is examined. We recently identified an equilibrium that exists between a transmembrane conformation of the T domain and a conformation that lies close to the cis surface of the bilayer, and introduced fluorescence methods to evaluate the amount of transmembrane insertion (*19*). Using these methods, we find that the E349K/D352K mutation significantly reduces the degree of transmembrane insertion of the T domain, and that the reduction in transmembrane insertion is correlated with a reduction in pore formation. However, it is possible for this mutant to undergo transmembrane insertion, and it can be shown that the protonation of other residues also must play an important role in promoting transmembrane insertion. We conclude that TH8/TH9 helix tip neutralization is only one factor favoring proper membrane insertion.

EXPERIMENTAL PROCEDURES

Materials. Spin-labeled PCs (12SLPC, 5SLPC, and TempoPC),[1] dioleoyl PC (DOPC), and dioleoyl PG (DOPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Lipid concentrations were determined by dry weight. The nitroxide content of spin-label PCs was determined as previously described (*16*). Rabbit anti-BODIPY-FL IgG, *N*-[(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyl]iodoacetamide (BODIPY-FL C1 IA, BODIPY-iodoacetamide), Cascade Blue (CB) labeled dextran (molecular weight 3000), anti-CB antibodies, and monochlorobimane were purchased from Molecular Probes (Eugene, OR). Isopropylthiogalactopyranoside was from Jersey Labs (Livingston, NJ). All other chemicals were of reagent grade. Precast polyacrylamide gels were purchased from Pharmacia Biotech (Piscataway, NJ). Synthetic oligonucleotides were ordered from Life Technologies (Rockville, MD). Vent polymerase was purchased from Biolabs (Beverly, MA).

Site-Directed Mutagenesis. The diphtheria toxin transmembrane domain DNA cloned into the pET-15b-T plasmid was used as the template for mutagenesis (*9*). Three primers were used for each mutagenesis reaction. The mutant primer contained the mutation site, and the two end primers were complementary both to the ends of extended T domain sequence and the *Eco*RI and *Nde*I cloning sites. Two step (asymmetric) PCR was used to introduce mutations into T domain DNA (*20*). In the first step, PCR was performed on a Perkin-Elmer GeneAmp PCR System using the primer containing the sequence being mutated and the primer overlapping the 3′ end of the T domain insert. In the second step, PCR was done with the product of the first reaction and the primer on the 5′ side of the T domain insert. After each step, the PCR products were purified by agarose gel electrophoresis. The final PCR product was cleaved by *Eco*RI and *Nde*I and inserted into the pET-15b vector (*21*). This plasmid was used to transform *Escherichia coli* strain DH5α. The T domain insertions were sequenced to confirm that only the proper mutations were introduced (Center for the Analysis and Sequence of Macromolecules, SUNY, Stony Brook, NY). Sequencing from both ends of the T domain DNA was performed covering the full T domain DNA region. No random mutations were detected. The pET-15b-T was then used to transform the *E. coli* BL 21 cell strain used for protein purification.

Protein Purification. Isolation of T domain from *E. coli* strains expressing the T domain mutants A356C (psuedo "wild-type") and E349K/D352K/A356C ("349K/352K") was performed basically as described previously (*9*). [These T domain constructions contain the hexaHis-tag-containing peptide followed by a tetrapeptide linker (GSHM) attached to T domain residues 202−378 (*9*).] Instead of a nickel-charged His binding, a Talon metal affinity resin was used (Clontech, Palo Alto, CA). A column containing about 1.5 mL of resin was washed with 1 mL of 0.25× wash buffer (wash buffer was 90 mM imidazole, 0.5 M NaCl, and 20 mM Tris-Cl, pH 8), then 1 mL of 0.5× wash buffer, 1 mL of 0.75× wash buffer, 1 mL of 1× wash buffer, and then a series of individual 1 mL aliquots of elution buffer (1 M imidazole, 0.5 M NaCl, and 20 mM Tris-Cl, pH 8). The T domain tended to elute in the last wash buffer and first two elution buffer aliquots. The T domain fractions were combined, diluted to 50 mL with 20 mM Tris-Cl, pH 8, and then subjected to FPLC on a 1 mL Source-Q anion-exchange column (Pharmacia Biotech, Piscataway, NJ), eluting at a rate of 0.5 mL/min with a 0−500 mM NaCl gradient containing 20 mM Tris-Cl, pH 8. The T domain eluted at about 250 mM NaCl. The purified fractions (detected by SDS gel electrophoresis) were combined at stored at 4 °C. The His_6 tag on the T terminal was not removed (*19*). T domain concentration was determined from the absorbance at 280 nm using $\epsilon = 18\,200\ M^{-1}\ cm^{-1}$, and converted to micrograms using an approximate molecular weight of 20 000. Protein purity was tested on polyacrylamide gels using a Phastsystem (Pharmacia Biotech) visualizing protein with Coomassie Blue staining. Final purity appeared to be >95%.

Bimane and BODIPY Labeling of T Domain. T domain proteins were labeled by bimane and BODIPY as previously described for the A356C mutant (*19*). A sample of wild-type T domain protein (lacking the A356C mutation) was labeled in parallel as a control. The fluorescence emission intensity of the labeled T domain was measured and it was found that the Cys-containing mutant had at least 10 times higher fluorescence than the Cys-less wild-type. The labeling of A356C and E349K/D352K/A356C was the same as shown by their having the same intensity of bimane and BODIPY fluorescence per microgram of protein. The recovery of protein was assumed to be nearly quantitative (*19*).

Fluorescence Measurements. Fluorescence was measured at room temperature with a Spex Fluorolog τ2 spectrofluorometer operating in the ratio mode. Unless otherwise noted, measurements were made in a semimicro cuvette (excitation path length 10 mm, emission path length 4 mm). The excitation and emission slit widths used were 1.5 and 5 mm, respectively. Trp emission spectra were measured with excitation at 280 nm. Excitation wavelengths of 370 and 480 nm were used for bimane, and BODIPY-labeled proteins,

[1] Abbreviations: BODIPY-iodoacetamide *N*-[(4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-*s*-indacene-3-yl)methyl]iodoacetamide; DMoPC, di $C_{14:1\Delta 9c}$phosphatidylcholine; DOPC, di $C_{18:1\Delta 9c}$phosphatidylcholine; DOPG, di $C_{18:1\Delta 9c}$phosphatidylglycerol; 5 or 12 SLPC, 1-palmitoyl-2-(5- or 12-doxyl)stearoyl phosphatidylcholine; PC, phosphatidylcholine; and TempoPC, 1,2-dioleoyl-*sn*-glycero-3-[4-(*N*, *N*-dimethyl-*N*-(2-hydroxyethyl)ammonium]-2,2,6,6-tetramethylpiperidine-1-oxyl).

respectively. Emission spectra of bimane-labeled T domain were scanned at 0.5 nm intervals from 440 to 480 nm at a rate of 0.5 nm/s for bimane-labeled proteins. An emission wavelength 516 nm was used to measure BODIPY fluorescence. An excitation wavelength of 385 nm and emission wavelength of 418 nm were used measuring the fluorescence of Cascade Blue (CB) labeled dextrans. Cascade Blue and BODIPY fluorescence was measured for 3 s. For bimane, BODIPY, and Cascade Blue measurements, background intensities and/or spectra from samples containing unlabeled protein or lacking labeled dextran, as appropriate, were subtracted. All experiments were done at room temperature (about 23 °C) unless otherwise noted. Control pH titration experiments showed no effect of temperature on the degree of deep insertion between 20 and 30 °C.

Vesicle Preparation. For most model membrane containing samples, ethanol dilution small unilamellar vesicles (SUV) composed of 30% DOPG/70% DOPC (mol/mol) were used. SUV were prepared as previously described (*22*).

Purification of CB-Labeled Dextrans. To obtain dextrans of relatively homogeneous size, the commercial dextrans were fractionated using gel filtration chromatography. CB-dextran (1 mL of 5 mg/mL, 3 kDa) was loaded on a Sephacryl S-200 column (0.5 cm × 40 cm) and eluted with PBS, pH 7 (*22*), at room temperature. Fractions of 1 mL were collected. Dextran elution was monitored by CB fluorescence. The four peak fractions were pooled, and 1 mL of each pool was rechromatographed. The two peak fractions from the rechromatography step were pooled (2 mL total) and stored at 4 °C.

Entrapping Dextrans within Model Membrane Vesicles. The CB-labeled dextrans (3 kDa) were trapped inside large unilamellar vesicles (LUVs) formed by octyl glucoside dialysis (*23*) at room temperature. The LUVs were composed of 30% DOPG/70% DOPC. Dialysis tubing (Spectra/Por) with a molecular weight 1000 cutoff was used. Vesicles contained 0.02 mol % rhodamine-PE as a fluorescent marker used to assay lipid concentration. Prior to dialysis, total lipid concentration was 10 mM. Initial dextran concentrations were approximately 0.03 mM. Free dextran was separated from vesicle-trapped dextran by filtration on a Sepharose 4B-CL gel filtration column (0.5 cm × 40 cm) at room temperature. Samples were eluted from the column with PBS, pH 7.2, and the fractions containing lipid were pooled. In some preparations, dextran concentration was monitored to determine trapping efficiency. (Dextran concentrations were determined using a CB fluorescence vs concentration curve.) From CB fluorescence measurements, trapping efficiencies were found to be between 5% and 7%. The final concentration of lipid was 2−4 mM, and the final concentrations of the dextrans were approximately 0.5 μM.

Dextran-containing vesicles were stored in the PBS 7.2 buffer at 4 °C. The CB-dextrans remained entrapped inside the LUVs for a period of 2 weeks as determined by the lack of CB quenching (<5%) upon antibody addition in the absence of toxin.

Effect of Increasing T Domain Concentration on Bimane Fluorescence. To examine the effect of T domain concentration on the bimane fluorescence of vesicle bound T domain molecules (i.e., wild-type or 349K/352K), an aliquot of up to 10 μL containing 0.5 μg of bimane-labeled T domain in PBS was added to 30% DOPG/70%DOPC ethanol dilution vesicles prepared in 580 μL of 10 mM sodium acetate/150 mM NaCl, pH 4.5 (acetate buffer). The resulting lipid concentration was 200 μM and volume 600 μL. To this sample, 2−7 μL aliquots of the corresponding (i.e., wild-type or 349K/352K) but unlabeled T domain molecule in PBS were then added. After each aliquot the samples were incubated about 15 min and then fluorescence was remeasured. The pH remained at 4.5 throughout.

Kinetics of Concentration Dependence Effects on Bimane Fluorescence. To look at the kinetics of the concentration effects, samples containing 0.5 μg of bimane-labeled T domain (mutant or wild-type, respectively) were prepared as described above. Each sample was placed in a cuvette, mixed continuously, and fluorescence measured at 450 and 470 nm over a 1−2 s interval every 15 s. At the desired time point, 9.5 μg of the same T domain molecule as already in the sample (mutant or wild-type, but unlabeled) was added and fluorescence measurements at 450 nm and 470 nm were continued for the desired period.

Effect of pH on Bimane Fluorescence. For experiments in which the effect of pH on the fluorescence of membrane-bound bimane-labeled mutant T domain protein was examined, samples were prepared as described in the experiments in which T domain concentration was varied, except that the acetate buffer used was at pH 6. Aliquots (about 3−5 μL) of various concentrations of acetic acid or (below pH 4) HCl were added to the samples and mixed gently. Fluorescence was measured 15 min after each aliquot was added.

Kinetics of the Release of CB-Dextrans from Vesicles. Dextran release kinetics were performed by diluting an aliquot (30 μL) of vesicles containing entrapped CB-labeled dextran and a 4 μL aliquot from a 2.5 mg/mL stock solution of anti-CB antibodies to 660 μL with pH 4.5 acetate buffer (final pH remained 4.5). Fluorescence was measured at 15 s intervals after 5 μg of T domain was added and mixed gently. Fluorescence measurements were then continued for 15 min.

Pore Formation vs T Domain Concentration. Samples were prepared by mixing an aliquot (30 μL) of vesicles containing entrapped CB-labeled dextran with 4 μL from a 2.5 mg/mL stock solution of anti-CB antibodies and diluting to 660 μL with, pH 4.5, acetate buffer (final pH remained 4.5). Fluorescence was measured, and then 3−10 μL aliquots of the appropriate unlabeled T domain added, with the samples incubated for 15 min after each aliquot of toxin was added before fluorescence was remeasured.

Pore Formation vs pH. Samples were prepared by mixing an aliquot (30 μL) of vesicles containing entrapped CB-labeled dextran with 4 μL from a 2.5 mg/mL stock solution of anti-CB antibodies and diluting to 660 μL with pH 6 acetate buffer. Then 6.5 μL (containing 5 μg) of psuedo wild-type T domain or 5.5 μL (containing 5 μg) 349K/352K mutant (see Results) was added. Fluorescence was measured after 15 min and then aliquots (about 3−5 μL) of various concentrations of acetic acid or HCl (below pH 4) was added to the samples and mixed gently. Fluorescence was measured 15 min after each aliquot was added.

Fluorescence Quenching of Bimane-Labeled T Domain Mutants. For fluorescence-quenching experiments, ethanol

dilution vesicles with 30% DOPG/70% PC (DOPC with or without 15% nitroxide labeled lipid, 12SLPC, 5SLPC, or TempoPC)[2] were prepared similarly to those described previously (*19*). The mixed lipids dissolved in 10 μL of ethanol were diluted with at least 575 μL of acetate buffer, pH 4.5, and then an aliquot of T domain to bring the volume to 600 μL. The final samples contained 200 μM lipid. Fluorescence was then measured (after a 15 min incubation) at excitation 370 nm and emission 465 nm. To make measurements at pH 3, an aliquot of concentrated HCl was added to each sample, and after a 15 min incubation, fluorescence was remeasured.

Quenching by Anti-BODIPY Antibodies. The initial samples were prepared similarly as described for the initial samples for experiments in which the effect of increasing T domain concentration on bimane fluorescence was measured except a 20 μL aliquot of BODIPY-labeled T domain, and 570 μL of acetate buffer, pH 4.5, was used. Fluorescence was measured, and then 40 μL of an anti-BODIPY stock solution (4 mg/mL) (at pH 4.5) or 80 μL (at pH 3) was added to the samples (19). After 15 min, fluorescence was remeasured two times. For samples with excess unlabeled protein, a small aliquot (5−20 μL) containing 4 μg of unlabeled wild-type or 349K/352K mutant T domain was added immediately after the labeled wild-type or 349K/352K mutant, respectively, and before the anti-BODIPY antibody.

RESULTS

Bimane Fluorescence Shows That the 349K/352K Mutant Has a Reduced Tendency To Undergo Transmembrane Insertion. Our previous studies demonstrated that a bimane label attached to 356C is sensitive to T domain orientation within the membrane (*19*). Bimane fluorescence detects two orientations that penetrate the membrane to different degrees. In the more deeply inserting transmembrane (TM) state,[3] bimane emission blue shifts (i.e. shifts to shorter wavelengths), whereas in the nontransmembrane (partially penetrating, or P) state, it exhibits red shifted fluorescence. That these wavelength shifts reflect different degrees of membrane penetration was confirmed by fluorescence quenching and antibody binding (*19*). Therefore, to examine the effect of the E349K and D352K mutations on the transmembrane insertion of the T domain, their effect on the fluorescence of bimane-labeled Cys356 was compared to that of bimane-labeled protein that is wild-type at residues 349 and 352. T domain carrying the E349K, D352K, and A356C mutations is referred to as the 349K/352K mutant. The wild-type protein is actually a psuedo wild-type because it carries the labeled 356C residue, but we will refer to it as wild-type for simplicity.

In the previous study, it was shown that for wild-type T domain there is a blue shift (from about 466 nm to about 459 nm)[4] as T domain concentration is increased, due to conversion of the T domain in the P conformation to the TM conformation (*19*). Comparing the effect of T domain concentration on the bimane emission of the 349K/352K mutant and wild-type protein (Figure 1) shows that the mutant clearly has a lesser tendency to form the TM conformation, as judged from the lesser degree of blue shift relative to the wild-type as T domain concentration is increased. However, from Figure 1, it also appears that at high T domain concentrations even the mutant can acquire the TM conformation (also see below). A similar reduced tendency for the mutant to form the TM conformation was also observed in experiments in which the unlabeled wild-type protein was added to bimane-labeled 349K/352K mutant (not shown).

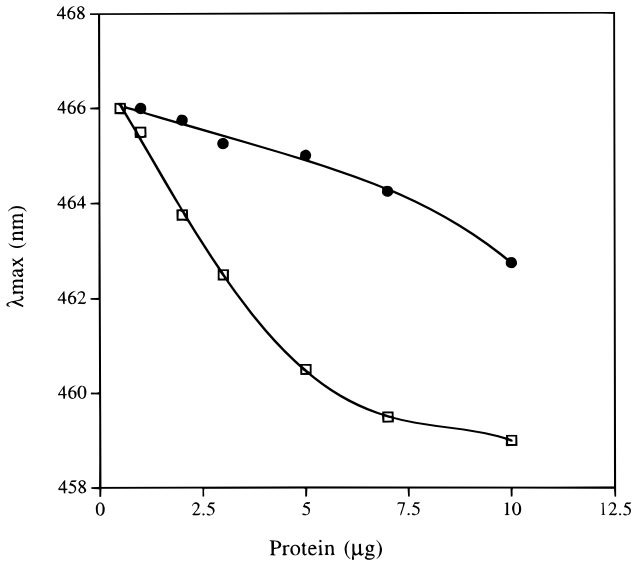

FIGURE 1: The effect of T domain concentration on bimane emission of wild-type and 349K/352K Mutant. Samples contained 0.83 μg/mL bimane-labeled (□) wild-type or (●) bimane-labeled 349K/352K mutant incorporated in 600 μL of 200 μM 30% DOPG/70%DOPC SUV at pH 4.5. To these aliquots containing the indicated amounts of wild-type or 349K/352K mutant, respectively, were added. The reproducibility of duplicate experiments was about ±1 nm.

To determine if the difference between wild-type and 349K/352K mutant reflected a difference in the rate of insertion, the kinetics of insertion was examined. To do this, bimane-labeled protein was bound to vesicles under conditions in which both the wild-type and 349K/352K mutant do not insert in the transmembrane conformation (low protein concentration, 0.5 μg/per sample), and then 9.5 μg of the corresponding unlabeled T domain was added to induce the formation of the transmembrane conformation. In both cases deeper insertion, detected by a blue shift monitored by an increase in fluorescence at 450 nm relative to that at 470 nm, exhibited $t_{1/2} < 15$ s (Figure 2). Since mixing is likely to be rate limiting, the actual time for insertion may be more rapid that this. An insertion time at least this rapid is also shown by the observation that both wild-type and 349K/352K mutant form pores on this time scale (see below).

Control experiments indicated that the difference between the wild-type and 349K/352K mutant was not due to misfolding of the 349K/352K mutant. Low UV circular

[2] Spin-labeled PCs contain a certain amount of inactive (nonquenching) label. The 55% remaining PC is a mixture of the inactive label and DOPC.

[3] Experiments on a series of residues in TH8 and TH9 have revealed that residue 349 is located close to the trans surface in the TM state, and thus the TM state is fully transmembraneous (*28*).

[4] All wavelength values are about 3 nm smaller than previously reported (*19*) due to the fact that the Spex τ2 has a different wavelength response than the Spex 212 used previously. Controls in which the samples were read on both instruments confirmed this instrumental artifact.

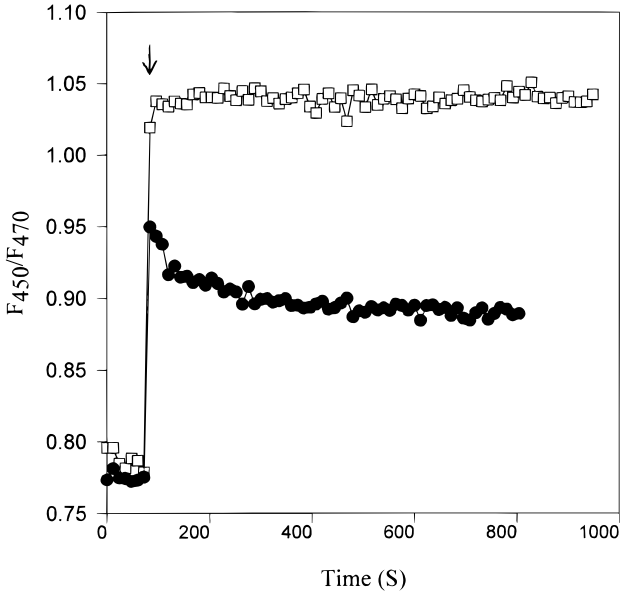

FIGURE 2: Kinetics of deep insertion of bimane-labeled T domain. Samples contained 0.83 μg/mL (□) wild-type or (●) 349K/352K mutant incorporated into 600 μL 200 μM 30% DOPG/70%DOPC SUV at pH 4.5. To initiate deep insertion at the time shown by the arrow 9.5 μg of the corresponding unlabeled T domain was added, and fluorescence was measured at 450 and 470 nm at various times.

dichroism indicated a similar α-helix-rich secondary structure for the mutant and wild-type protein in solution at pH 7, in agreement with the crystal structure of T domain in whole toxin (*2*). As judged by nearly identical Trp and bimane emission wavelengths, the wild-type and 349K/352K mutant also have similar tertiary structures at pH 7 in solution. Furthermore, the stability of the folding of the wild-type and 349K/352K mutants in solution at pH 7 was also similar, as denaturation by guanidinium-Cl occurred over the same concentration range, with a midpoint at 1.5−2 M, as judged by the loss of secondary structure detected by low UV circular dichroism and by shifts in both Trp and bimane fluorescence emission to longer wavelengths (data not shown).

Decreasing pH Promotes Conversion of the P Conformation to the TM Conformation. In the course of these experiments, we discovered that the conformational equilibrium between the P and TM membrane-inserted states was pH dependent as well as protein concentration dependent (Figure 3). At low toxin concentration, the wild-type T domain is in the P conformation at pH 4.5 but can be converted to the TM conformation by decreasing pH to 3. This indicates that protonation of residues on the T domain at low pH not only promotes membrane association, but also transmembrane insertion.[5] Furthermore, the effects of low pH and high T domain concentration appear to be cumulative. At higher T domain concentrations, the pH necessary for full TM insertion is increased by about a full pH unit. This suggests that the promotion of transmembrane insertion by very low pH and high T domain concentration are closely linked.

The 349K/352K Mutant Is Transmembranous at Very Low pH, but Shows a Reduced Tendency To Take on the TM Conformation. Figure 3 also compares the effect of pH on the wild-type and 349K/352K mutant. With 5 μg of T domain, a lower pH (>0.5 units) is needed to induce the 349K/352K mutant to become transmembraneous than to induce the wild-type protein to become transmembraneous. This means it is more difficult to insert the mutant protein in a transmembrane conformation. However, at very low pH, the 349K/352K mutant does appear to fully take on the TM conformation as judged by its achieving a blue shift that is similar to that of the wild-type protein. This suggests that the introduction of Lys residues does not result in an absolute block to transmembrane insertion.

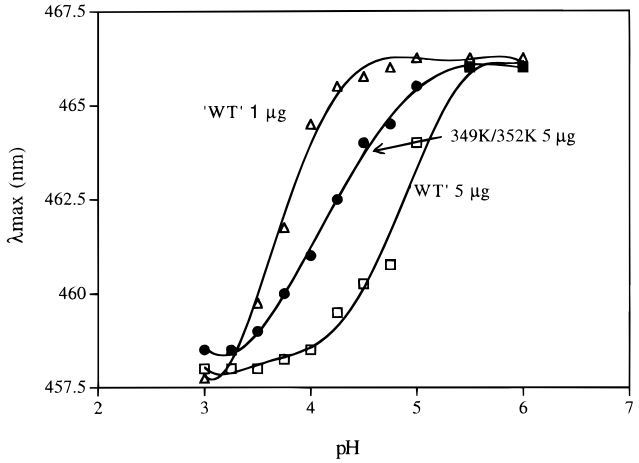

FIGURE 3: The effect of pH on bimane emission of wild-type and 349K/352K mutant. Samples initially contained 0.17 μg/mL (△) or 0.83 μg/mL bimane-labeled (□) wild-type or 0.83 μg/mL (●) 349K/352K mutant incorporated in 600 mL of 200 μM 30% DOPG/70%DOPC SUV. Sample pH was then decreased progressively by titration. The reproducibility of duplicate experiments was about ±1 nm.

The Differences between Wild-Type and 349K/352K Mutant Insertion Can Be Confirmed by Antibody Binding. The conclusions from the behavior of bimane-labeled T domain were confirmed by evaluation of the exposure to external solution of a BODIPY group attached to residue 356. We previously demonstrated that the exposure of the BODIPY group to solution can be monitored by the binding of anti-BODIPY antibodies, which quench BODIPY fluorescence upon binding, and showed that BODIPY exposure of residues that become buried in the TM conformation is less than in the P conformation (*19*).

Table 1 summarizes anti-BODIPY quenching of wild-type and 349K/352K mutant BODIPY labeled on residue 356. At pH 4.5, the wild-type protein shows more reactivity at low concentrations than at higher concentrations. This agrees both with previous results (*19*) and with the data in Figure 1, showing that residue 356 inserts more deeply at higher T domain concentrations. In contrast, at pH 4.5 BODIPY attached to the 349K/352K mutant remains relatively exposed to anti-BODIPY at higher T domain concentrations, in agreement with the relative lack of bimane blue shift for the mutant at higher concentrations (Figure 1). At pH 3, loss of anti-BODIPY binding indicates there is deep insertion even at low concentrations of T domain both for the wild-type and for the 349K/352K mutant (in agreement with Figure 3).[6] This supports the conclusion from bimane fluorescence that at very low pH both the wild-type and mutant undergo deep insertion.

[5] Control experiments using vesicles containing only zwitterionic PC also showed increased TM insertion at low pH (not shown). This rules out effects on T domain transmembrane insertion due to DOPG protonation at very low pH.

Table 1: Insertion of BODIPY-Labeled T Domain Monitored by Anti-BODIPY Antibody Binding[a]

protein	condition	% quenching
wild-type	1 μg, pH 4.5	44 ± 1%
wild-type	1 μg, pH 3[b]	23 ± 2%
wild-type	5 μg, pH 4.5	28 ± 5%
349K/352K	1 μg, pH 4.5	45 ± 1%
349K/352K	1 μg, pH 3	22 ± 2%
349K/352K	5 μg, pH 4.5	39 ± 6%

[a] Previous studies have shown about 50% quenching for membrane bound T domain residues fully exposed to the external aqueous solution and about 25% quenching for buried residues. The residual reactivity appears to be due to incomplete insertion. The maximal quenching obtained with a free BODIPY molecule was 74% (*19*). The results shown are the average of four individual determinations. Standard deviations are shown. [b] Anti-BODIPY binding to a free BODIPY probe was only marginally weaker at low pH. A higher antibody concentration was used to maintain saturating antibody levels (see Experimental Procedures).

Fluorescence Quenching by Nitroxide-Labeled Lipids Shows the Movement of Bimane-Labeled 356 to a Nonpolar Environment Correlates with Increased Depth in the Bilayer. To directly demonstrate the change in the location of residue 356 detected above corresponds to its becoming more deeply inserted in the membrane, quenching by nitroxide (spin)-labeled lipids was used. To do this, the quenching of the bimane fluorescence of labeled T domain by lipids carrying a shallow (TempoPC), medium (5SLPC), or deep (12SLPC) nitroxide was compared. A deep or shallow location of a fluorescent group is indicated when the quenching is strongest by the deep or shallow nitroxide, respectively.

Table 2 shows nitroxide-labeled lipid quenching of the wild-type and 349K/352K mutant. At pH 4.5 and low T domain concentrations (1 μg/sample), bimane attached to residue 356 on both the wild-type and 349K/352K mutant locates shallowly, as shown by the fact that the shallow TempoPC nitroxide quenches most strongly. In contrast, at pH 3, the bimane on both the wild-type and 349K/352K mutant locates more deeply, as shown by the observation that the deep nitroxide of 12SLPC quenches most strongly. This is in agreement both with the bimane emission wavelength shift data and the BODIPY exposure results.

In addition, the quenching data at higher T domain concentrations at pH 4.5 (Table 2) shows that both for the wild-type and 349K/352K mutant strongest quenching is obtained with the deep 12SLPC. This confirms that insertion at high T domain concentrations is deeper than that observed at low T domain concentrations. However, the depth of the bimane label appears to be less in the case of the 349K/352K mutant at high T domain concentrations. This can be seen by the fact, that for the wild-type, quenching by 12SLPC was much stronger than that by TempoPC, whereas for the 349K/352K mutant, quenching by the 12SLPC was only moderately stronger than for the TempoPC. This agrees with the smaller loss of anti-BODIPY antibody binding and smaller blue shift seen for the 349K/352K mutant as compared to the wild-type under high T domain concentration conditions (see above).

[6] Control experiments with free BODIPY-iodoacetimide showed that the lesser quenching at pH 3 was not due to weaker antibody binding. Only slightly weaker binding to BODIPY-iodoacetimide was found at pH 3 relative to pH 4.5 and to compensate, 2-fold more anti-BODIPY was added at pH 3.

The Ability of the T Domain To Form Pores Is Closely Linked to Its Transmembrane Insertion. The dependence of pore formation by the wild-type protein and the 349K/352K mutant was compared to study the relationship of deep insertion and pore formation. To examine pore formation, the release of 3K Cascade Blue labeled dextrans from vesicles was measured. In this assay, the release of dextran is detected by the quenching of Cascade Blue fluorescence by anti-Cascade blue antibodies in the external solution.

Dextran release experiments demonstrated release of 3K dextran that was dependent on the concentration of T domain present (Figure 4). The release of dextran by the 349K/352K mutant was less than that by the wild-type protein at all concentrations. The kinetics of release was similar ($t_{1/2}$ < 30−60 s) (Figure 5) for both the wild-type and the 349K/352K mutant, so the difference in the amount of dextran they release must reflect a difference in the size or number of pores formed rather than the rate of release through the pores.

The amount of dextran released from the vesicles was also found to be dependent on pH. At pH 4.5, where the 349K/352K mutant shows less TM insertion than wild-type, it also showed less 3K dextran release. As pH was decreased from pH 4.5 to pH 3, the degree of dextran release progressively increased for both wild-type T domain and the 349K/352K mutant (Figure 6). The increase in release occurred at a higher pH for the wild-type protein than the mutant. This correlates well with the difference in deep insertion of mutant and wild-type as a function of pH. Below pH 3.5, where bimane fluorescence and antibody binding indicate deep insertion of both wild-type and mutant, the wild-type and 349K/352K mutant released a similar amount of 3K dextran. Thus, the formation of pores large enough to release the 3K dextran is closely connected to the transition from the P conformation to the TM conformation. There is also evidence for an intermediate plateau in dextran release in the pH region 3.5−4.5. We are not certain of the origin of this plateau, but it may reflect a subtle difference in the structure or amount of deeply inserted conformation at pH 3 and at pH 4.

DISCUSSION

Residues E349 and D352 Play a Significant but Not Essential Role in Transmembrane Insertion in Vitro. The details of the process of membrane penetration by toxins and other membrane proteins is little understood. One key question is how ionizable residues that must cross a membrane affect the insertion process. The role of protonation of acidic residues within diphtheria toxin in membrane insertion has been long recognized (*2*, *6*, *24*, *25*), and it has been proposed that protonation of acidic residues (i.e., a loss of charge) at the tips of helices that become transmembraneous would be critical for triggering efficient and rapid transmembrane insertion (*2*, *16*). In agreement with a role for helix tip neutralization, previous studies have shown that mutations affecting charge at residues E349 and D352, which are in the loop between the tips of helices TH8 and TH9, inhibit pore formation and toxicity (*16*, *18*). However, they did not distinguish between such mutants having a transmembrane conformation but unable to form a pore (or in which the pore is almost always closed) and mutants that do not form a pore because they are in a nontransmembraneous conformation.

Table 2: Depth of Bimane Insertion Monitored by the Fluorescence Quenching Induced by Nitroxide-Labeled Phospholipids

protein	condition	F_{TC}/F_o	F_5/F_o	F_{12}/F_o	$F_{TC}/F_o - F_{12}/F_o$[b]
wild-type	1 μg, pH 4.5	0.48 ± 0.03	0.61 ± 0.09	0.56 ± 0.04	−0.08 ± 0.05
wild-type	1 μg, pH 3	0.66 ± 0.08	0.67 ± 0.08	0.56 ± 0.04	+0.12 ± 0.02
wild-type	5 μg, pH 4.5	0.80 ± 0.09	0.68 ± 0.09	0.51 ± 0.05	+0.29 ± 0.09
349K/352K	1 μg, pH 4.5	0.51 ± 0.02	0.65 ± 0.02	0.57 ± 0.02	−0.06 ± 0.03
349K/352K	1 μg, pH 3	0.68 ± 0.02	0.71 ± 0.04	0.60 ± 0.02	+0.08 ± 0.03
349K/352K	5 μg, pH 4.5	0.60 ± 0.03	0.65 ± 0.02	0.54 ± 0.02	+0.07 ± 0.04

[a] F/F_o is the ratio of fluorescence in the presence of lipid vesicles containing the shallow TempoPC (F_{TC}), medium depth 5SLPC (F_5) or deep 12SLPC (F_{12}) quenchers to than in vesicles lacking quencher (F_o). The values shown are the average of four determinations, the standard deviation of which is also shown. For the wild-type the absolute quenching levels are slightly different than previous values but the same qualitative changes due to increased T domain concentration was observed (*19*). [b] These values were not determined from the average values in the preceding column, but rather from the difference in values for experiments done on the same day. The difference between the values at pH 4.5 with 1 μg of protein and the other values are statistically significant, with $p < 0.01$ in all cases.

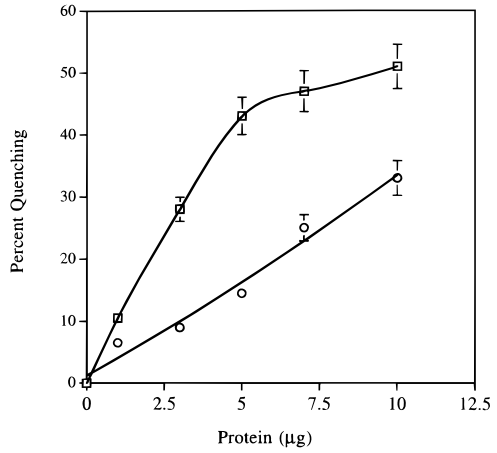

FIGURE 4: The effect of T domain concentration on pore formation by wild-type and 349K/352K Mutant. Samples contained unlabeled (□) wild-type or (○) 349K/352K mutant added to 200 μM 30% DOPG/70%DOPC LUV into which 3 kDa Cascade Blue labeled dextran had been entrapped, at pH 4.5. The percent quenching by anti Cascade-Blue antibodies added externally was measured 15 min after the addition of each aliquot of T domain (see Experimental Procedures for details). The range of duplicate experiments is indicted by the vertical bars.

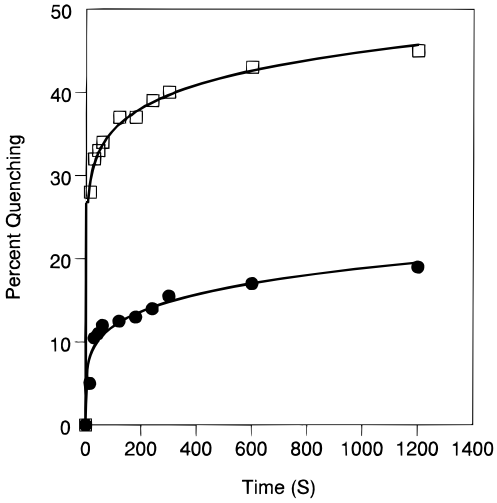

FIGURE 5: Kinetics of T domain release of entrapped 3 kDa Cascade Blue labeled dextrans. Samples contained (□) wild-type or (●) 349K/352K mutant added to 200 μM 30% DOPG/70%DOPC LUV within which 3 kDa Cascade Blue labeled dextran had been entrapped, at pH 4.5. The percent quenching by anti Cascade-Blue antibodies added externally was measured various times after the addition of T domain.

This study shows that these mutations inhibit transmembrane insertion. However, the inhibition is not absolute, and it is possible for the 349K/352K mutant to acquire a

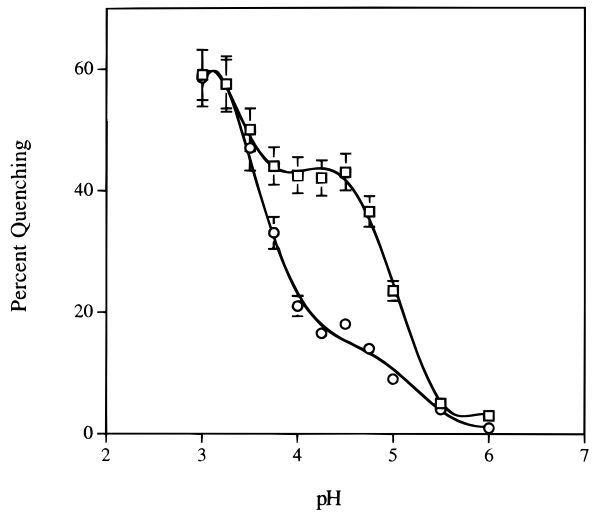

FIGURE 6: The effect of pH on pore formation by wild-type and 349K/352K mutant. Samples contained 5 μg of unlabeled (□) wild-type or (○) 349K/352K mutant incorporated in 200 μM 30% DOPG/70%DOPC LUV into which 3 kDa Cascade Blue labeled dextran had been entrapped. pH was decreased gradually by titration. The percent quenching by anti Cascade Blue antibodies added externally was measured 15 min after each adjustment of pH. The range of duplicate experiments is indicted by the vertical bars. Percent quenching is given relative to initial fluorescence at pH 6 prior to antibody addition. Cascade Blue fluorescence was pH-independent between pH 3 and 6 in the absence of antibody and T domain.

transmembrane conformation (Figure 7). We conclude that the presence of two charged residues that must almost totally cross the bilayer can be insufficient to block transmembrane insertion of a protein. Furthermore, under favorable conditions, deep insertion of the mutant proteins is rapid, although the actual rate of insertion was too fast to be detected. Since charged small molecules can only cross lipid bilayers slowly and since residues 349 and 352 are exposed at the tips of helices, a larger effect of their being charged on the kinetics of insertion would have been predicted. Perhaps these residues interact with other polar groups on T domain residues in such a way that they are not fully exposed to the hydrophobic acyl chains of lipids, and/or are not protonated, during the translocation process.

Why Does Mutation to Lys Inhibit Transmembrane Insertion Weakly? The observation that there is no total kinetic block to insertion raises the question of why Lys residues at positions 349 and 352 inhibit transmembrane insertion to any degree. The possible effects of introducing a basic residue into the sequence of a membrane-inserting helix on the stability of transmembrane inserted state at low pH

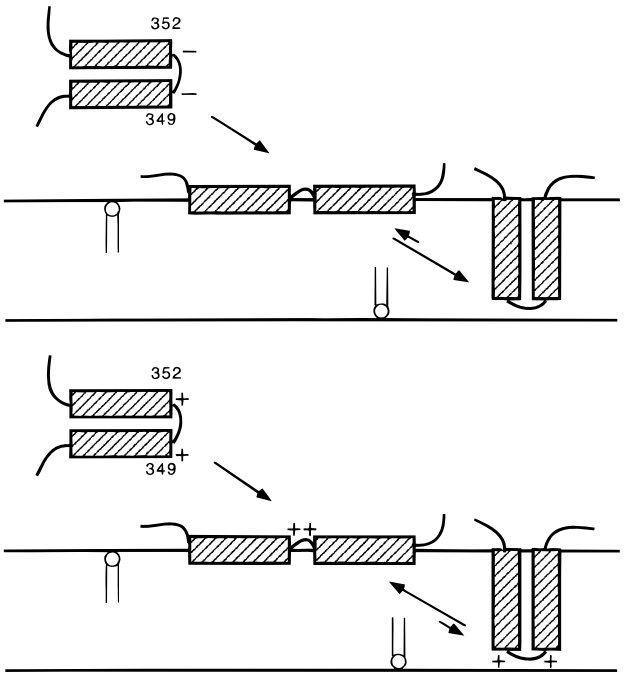

FIGURE 7: Schematic illustration of the difference between wild-type and 349K/352K mutant behavior. Only the TH8/TH9 region of the T domain is shown. The helices are represented by rectangles. The movement of the helices from solution to the bilayers represents the low pH triggered conformational change and its aftermath.

(relative to a state where the residues remain immersed in the aqueous solution on the cis side of the bilayer) should depend strongly on the location of the residue in the transmembrane state. In a hydrophobic environment the energy of a basic group could be very high relative to that of its charged state in solution. On the other hand, there should be little effect of such a residue if it remains totally immersed in aqueous solution after insertion. Residues 349 and 352 probably represent an intermediate case (Figure 7), as in the transmembrane conformation, residues 349 and 352 appear to locate close to the membrane surface on the trans side of the bilayer (*2*, *15*, *28*). Such a location could result in some degree of inhibition of transmembrane insertion by Lys substitutions, because even at the membrane surface, the energy of charged groups increases relative to aqueous solution, as exemplified by the increase in pK_a for membrane-bound fatty acid carboxyl groups relative to pK_a in solution (*26*). In contrast, the native acidic residues at these positions could remain protonated and uncharged at low pH more easily, and so result in an overall lower energy for the transmembrane form.

Of course, the energetic effects of a mutation on hydrogen bonding and other interactions with other residues within the protein must also be considered. For example, the effects of introducing a Lys at 349 and 352 could arise from the loss of interactions due to the removal of the carboxyl group rather than the effects of introducing a cationic amino group. However, Ala substitutions at E349 and D352 have already shown that there are only very small effects on pore formation and toxicity relative to those for the Lys substitutions (*12*, *16*), suggesting that the interactions of the Asp and Glu carboxyl groups themselves are not critical. Nevertheless, even if the charge on the Lys group is the source of destabilization of the transmembrane conformation, the location of the Lys relative to the lipid may not be its most critical effect. Because there is some evidence that T domain oligomerization aids transmembrane insertion, it is possible that destabilization of oligomerization by Lys is important (*19*, *28*).

If Lys substitutions on the lipid-exposed face of a membrane-inserting toxin are especially destabilizing, it may be possible to use a series of Lys substitutions to study helix topography. A simple pattern of insertion inhibition may occur for a systematic series of Lys substitutions along the exposed face of a helix. Such a study would be practical for helix TH9 where the face exposed at low pH has been revealed by ESR (*15*). In this regard, it is interesting that the I364K mutation, which involves a deeply inserted residue on the lipid exposed face of TH9, has been found to strongly inhibit toxicity and pore formation (*29*).

It is noteworthy that earlier studies found effects of these Lys mutations on both pore formation and toxicity that were larger than that observed in this study. One likely explanation of this difference is that the exact pH studied is critical for the degree of transmembrane insertion. In this study, we chose pH 3−4.5, because a significant fraction of the T domain can take on the transmembrane form at these pH values. However, previous experiments showing a large loss of toxicity and pore formation for these mutants examined membrane insertion at pH 5.0−5.3 and above. At these higher pH values, there is likely to be much less transmembrane insertion for both the wild-type and mutants, and the difference between the mutant and wild-type deep insertion and pore formation will be larger than at pH 4.5 (see Appendix). Since endosomal conditions more closely approximate pH 5, the role of 349 and 352 in transmembrane insertion in vivo is likely to be more important than indicated by our in vitro experiments. On the other hand, to study the transmembrane form in vitro, the lower pH 4.5 or 3 conditions are more useful and, in fact, apparently necessary in order to have the majority of molecules in the transmembraneous conformation.

Ionizable Residues in Addition to 349 and 352 Must Be Involved in Transmembrane Insertion. Another observation in this study is that very low pH can promote conversion of the shallow P conformation to the transmembrane (TM) conformation of the T domain. Since such an effect of pH must involve protonation of ionizable residues and can be observed even for the 349K/352K mutant, there must be T domain residues in addition to E349 and D352 whose protonation promotes transmembrane insertion. Two possibilities are residues D295 and D318. The studies of Falnes et al. (17) indicated that Lys mutations at D295 and D318 reduce pore formation and toxicity [although the study of Silverman et al. only found a strong effect in the case of the D295K mutant (*16*)].

It should also be noted there are potentially many other residues in the T domain whose protonation promotes transmembrane insertion, but which have not yet been detected by site-directed mutagenesis. Such residues are not necessarily restricted to acidic residues within loops that reach the trans side of the membrane. For example, they could be acidic residues within less hydrophobic helices. It is also conceivable that protonation of specific His residues at low pH stabilizes the transmembrane state. Further studies will be needed to identify such residues.

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APPENDIX: RELATIONSHIP BETWEEN pH AND MUTATIONAL EFFECTS ON TRANSMEMBRANE PROTEIN INSERTION

For a protein, such as a toxin, where pH controls membrane insertion, the effect of a mutation that alters the stability of the transmembrane state will be strongly pH dependent. Consider the case of a protein which has an ionizable residue (or residues), which when in the protonated form gives rise to the transmembraneous conformation, and which when ionized form gives rise to a shallower nontransmembraneous (P) conformation. That this model applies to the T domain can be deduced from the pH dependence of transmembrane insertion in Figure 3. For mathematical simplicity, consider the case in which the protonation of a single residue leads to transmembrane insertion, such that insertion is shallow (P conformation) when this residue is ionized and transmembraneous when this residue is protonated. Let T_{total} equal the total T domain concentration; T equal the concentration of T domain on which this residue is ionized (it is also the concentration of shallow T domain), HT equal the concentration of protonated T domain [it is also the concentration of transmembrane T domain ($T_{transmembrane}$)], K equal the ionization constant for the critical residue (it is also the pH where the T domain is half protonated and where half is in the transmembrane state), and H equal the proton concentration.

Then

$$K = (H)(T)/(\mathrm{HT}) = (H)(\mathrm{T}_{total} - \mathrm{HT})/(\mathrm{HT}) = [(H)(T_{total})/(\mathrm{HT})] - (H)$$

and solving for $(\mathrm{HT})/(T_{total})$ yields

$$(\mathrm{HT})/(T_{total}) = (H)/(K + H) \quad (1)$$

If eq 1 refers to wild-type T domain, then for a mutant having a different equilibrium constant (K') for the ionization of the critical residue,

$$(\mathrm{HT}_{mutant})/(T_{total}) = (H)/(K' + H) \quad (2)$$

and the ratio $[(\mathrm{HT})/(\mathrm{HT}_{mutant})]$ is given by the ratio of eqs 1 and 2:

$$(1)/(2) = (\mathrm{HT})/(\mathrm{HT}_{mutant}) = (K' + H)/(K + H)$$

Note that the ratio $(\mathrm{HT})/(\mathrm{HT}_{mutant})$ equals the ratio $(T_{transmembrane\ wild-type}/T_{transmembrane\ mutant})$.

We are concerned with the case in which the mutant undergoes the transition to the transmembrane conformation at a lower pH than the wild-type, i.e., $K' > K$, meaning the ionization constant for the mutant is higher than that of wild-type. At low pH (where $H \gg K,K'$), the ratio $[T_{transmembrane\ wild-type}/T_{transmembrane\ mutant}] = [(\mathrm{HT})/(\mathrm{HT}_{mutant})]$ equals 1, and at high pH (where $H \ll K,K'$), this ratio is a maximum. A 1 pH unit difference in the pH at which the wild-type and mutant are half-transmembraneous ($K'/K = 10$) would give a 10-fold difference in the amount of mutant and wild-type that are inserted transmembraneously at high pH. If there is cooperative protonation of two residues associated with the conformational change, then a 1 pH unit difference in the pH at which the wild-type and mutant are half-transmembraneous (which gives $K'/K = 100$) would increase the difference at high pH to 100-fold (calculation not shown).

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