Identification of Shallow and Deep Membrane-penetrating Forms of Diphtheria Toxin T Domain That Are Regulated by Protein Concentration and Bilayer Width

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Yang Wang‡, Susan E. Malenbaum‡, Kelli Kachel‡, Hangjun Zhan‡,†, R. John Collier§, and Erwin London‡†**

From the ‡Department of Biochemistry and Cell Biology and §Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11794 and the ¶Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

The α-helix-rich, hydrophobic transmembrane (T) domain of diphtheria toxin is believed to play a central role in membrane insertion by the toxin and in the translocation of its catalytic domain across membranes. In this report, T domain structure was studied using site-directed single-Cys mutants. The residues chosen, 322 (near the amino-terminal end of helix TH8), 333 (within helix TH8), and 356 (within helix TH9) were substituted with Cys and labeled with the fluorescent probe bimane. (Residues 333 and 356 should be located within the bilayer in the transmembrane state, and residue 322 should not penetrate the bilayer.) After insertion of T domain into model membrane vesicles, the location of bimane label relative to the lipid bilayer was characterized by its fluorescence emission and by its quenching with nitrooxide-labeled phospholipids. It was found that when the T domain is added to dioleoylphosphatidylcholine-containing vesicles, all three residues reside close to the outer surface. However, at high T domain concentration or in thinner dimysteol phosphatidylcholine-containing vesicles, a large fraction of residues 333 and 356 penetrate deeply into the membrane. In contrast, residue 322 remains exposed to aqueous solution under these conditions. These conclusions were confirmed by a novel antibody binding method. Antibodies that quench the fluorescence of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-3-indacene (BODIPY) groups were used to evaluate the exposure of BODIPY-labeled 322, 333, and 356. Maximum exposure of residues 333 and 356 to externally added antibody was only observed under conditions in which bimane fluorescence showed that these residues do not penetrate the bilayer. In contrast, residue 322 remained exposed under all conditions. We propose that the deeply penetrating T domain conformation represents a transmembrane or near-transmembrane state. The regulation of the transmembrane/nontransmembrane equilibrium should be a key to understanding diphtheria toxin membrane insertion and translocation. Our results suggest that toxin-toxin interactions may play an important role in regulating this behavior.

1 The abbreviations used are: ESR, electron spin resonance; BODIPY, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-3-indacene; BODIPY-iodoacetamide, N-((4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-3-indacene-3-yl)methyl)iodoacetamide; DMPC, diC14:1,2-rac-phosphatidylcholine; DOPC, diC16:1,2-rac-phosphatidylcholine; DOPG, diC16:1,2-rac-phosphatidylglycerol; 5SPLC and 12SPLC, 1-palmitoyl-2-(5-doxyl)stearoyl phosphatidylcholine and 1-palmitoyl-2-(12-doxyl)stearoyl phosphatidylcholine, respectively; PC, phosphatidylcholine; TempoPC, 1,2-dioleoyl-sn-glycero-3-(4,N,N-dimethyl-N-(2-hydroxyethyl)ammonium)-2,2,6,6-tetramethylpiperidine-1-oxyl; PG, phosphatidylglycerol.

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** To whom correspondence should be addressed: Tel. 516-632-8564; Fax. 516-632-8575; E-mail: elondon@ccmail.sunysb.edu.
† Present address: Arris Pharmaceutical, South San Francisco, CA 94080.
EXPERIMENTAL PROCEDURES

Materials—Spin-labeled PCs, dimyristoyl-PC (DMoPC), dipalmitytoyl-PC, dioleoyl-PC (DOPC), dioleosylenol-PC, dierucyl-PC, and dioleoyl-PC (DOPC) 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 described previously (18). Rabbit anti-BODIPY-FL IgG, N-(4,4-difluoro-5,7-dimethyl-4-benzo[c]xanthen-4-yl)methyl-dithioacetate (BODIPY-FL CI 1A, BODIPY-iodoacetamide), and monochlorobimane were purchased from Molecular Probes (Eugene, OR). All other chemicals were reagent grade.

Isolation of T domain from Escherichia coli strains expressing the T domain mutants H322C (322C), 1333C (333C), and A356C (356C) was performed as described previously (9). These T domain constructions contained a His6 tag followed by a tetrapeptide linker (GSHM) attached to T domain residues 202–378 (9). The protein without any mutations in the T domain sequence is referred to as wild type. Final purity appeared to be >95% as judged by SDS gel electrophoresis. Protein was stored at 4 °C in 20 mM Tris-C10 or 10 mM Tris-C10, 250 mM NaCl, pH 8.0. T domain concentration was determined from the absorbance at 280 nm using ε = 18,200 ± 1,000 M−1 cm−1 and converted to μg using an absorbance molecular weight of 20,000.

Fluorescence Measurements—Fluorescence was measured at room temperature with a Spex 212 Fluorolog spectrophotometer operating in the ratio mode. Unless otherwise noted, measurements were made in a semimicrocuvette (excitation path length 10 mm, emission path length 4 mm). The excitation and emission slit widths used were 2.5 and 5 mm, respectively. Trp fluorescence was measured with excitation at 280 nm. Emission intensity was generally measured at 330 and 350 nm for 4–8 s, or emission spectra were measured (at a rate of 1 nm/s). Bimane fluorescence was measured with excitation at 380 nm. Emission intensity was generally measured at 440 and 480 nm for 1–5 s, or emission spectra were measured. BODIPY fluorescence was measured for 20–30 s with excitation at 488 nm and emission wavelength at 516 nm. For Trp and bimane, background intensities and spectra from samples lacking protein were subtracted. Background values for BODIPY were negligible (<1%). Spectra were generally smoothed after subtraction.

Bimane Labeling of T Domain—For the 322C and 356C mutants, about 50 μM (25 mM) of mutant protein diluted to 1 ml with a Tris-C10NaCl, pH 8, buffer was dialyzed overnight against 1 liter of 10 mM Tris-Cl, 140 mM NaCl, pH 8.0. T domain concentration was determined from the absorbance at 280 nm using ε = 18,200 ± 1,000 M−1 cm−1 and converted to μg using an absorbance molecular weight of 20,000. Samples containing 5 μg/ml T domain were transferred into 1.4 ml of 10 mM Tris-C10, pH 8, by dialysis. Then 42 μl of 5% NaCl was added to bring NaCl concentration to 150 mM. The samples were then gradually titrated with 2–5 μl aliquots of 8 M acetic acid or NaOH to pH 4.6 and then with aliquots of 0.9 mM to achieve lower pH. For unlabeled protein, Trp fluorescence was measured after each addition; for bimane-labeled T domain mutants, bimane fluorescence intensity was measured after each addition. Readings were taken about 1 min apart. For these experiments, a 1-cm path length cuvette was used with 5-nm excitation and emission slits.

Effect of Acyl Chain Length on Bimane Fluorescence—Chloroform solutions of DOPG were mixed with DMoPC (14 carbon acyl chains), dipalmitytoyl-PC (16 carbon acyl chains), dioleosylenol-PC (20 carbon acyl chains), or dierucyl-PC (22 carbon acyl chains) in order to obtain a 30% DOPG, 70% PC (mol/mol) mixture (120 mM total lipid). The mixed lipids were dried under an N2 stream and then redissolved with 7.5 μl of ethanol. Vessicles were prepared by pipet dilution solution with 0.05 mM sodium acetate, 150 mM NaCl, pH 4.4 (17). Then 5 μg of labeled T domain mutant in 300 μl of Tris-C10, pH 8, was added, yielding a final pH 4.5. Final lipid concentration was 200 μM. Fluorescence intensity was then measured as described above.

Effect of Decane Addition on Bimane Fluorescence—To determine the effect of decane on the fluorescence of bimane-labeled T domain, samples containing DOPG/DMoPC or DOPG/DMoPC were prepared as described above. Aliquots of 1.9 (v/v) decane/ethanol were added. After a 1-min incubation, fluorescence intensity was measured. There was no effect of fluorescence seen in control experiments in which ethanol alone was added.

Effect of Increasing T Domain Concentration on Bimane Fluorescence—Bimane-labeled T domain mutants were added in 30% DOPG, 70% DOPC ethanol dilution vesicles prepared as described above, except using half the lipid and half the labeled T domain concentration to conserve materials. 2–6 μl aliquots of purified wild type T domain were then added. After a 30-s incubation, during which fluorescence restabilized, fluorescence was remeasured.

Fluorescence Quenching of Bimane-labeled T Domain Mutants—For fluorescence quenching experiments, lipid vesicles were prepared in a similar manner to that described above. Organic solvent solutions of DMoPC or DOPC, DOPG, and (when desired) spin-labeled PC were mixed. Samples containing 30 mol% DOPG and 70 mol% total PC. Samples containing spin-labeled PC, had 15 mol% of 12SPLC, 5SLPC, or TempoPC.2 The mixtures were dried under a N2 stream, redissolved in a few drops of CHCl3, and redried with N2. Then they were dissolved in 7.5 μl of ethanol and diluted with 300 μl of 335 mM sodium acetate, 150 mM NaCl, 3.5 mM Tris-C10, pH 4.4, to form vesicles. T domain dissolved in 300 μl of 10 mM Tris-C10, pH 8, was then added, to give a final pH of 4.5. Final lipid concentration was either 100 or 200 μM, and the amount of protein in the samples was 2.5 or 5 μg, respectively, to maintain a constant protein/lipid ratio. Emission spectra were scanned from 430 to 500 nm, and the fluorescence of samples with and without spin-labeled PC was recorded at the wavelength of maximum emission in the sample without quencher (i.e., 30% DOPG, 70% DMoPC or DOPC). For experiments in which the effect of protein concentration on quenching was measured, a small aliquot of a stock solution containing 10 μg of wild type T domain was then added to the samples. Fluorescence spectra were remeasured over a 15-min incubation period.

Quenching by Anti-BODIPY Antibodies—Anti-BODIPY experiments were done using sonicated small unilamellar vesicles prepared as described above for the labeling experiments, with the exception that labeling was performed after unfolding. For all three mutants, fluorescence measurements performed in the absence of anti-BODIPY antibodies were similar to those in vesicles made by sonication (see below), in which ethanol is not present. Specifically, with sonicated vesicles the same differences were seen between the emission wavelength of labeled T domains inserted into T domain vesicles, containing and DMoPC-containing vesicles, and at different protein/lipid ratios.

3 Spin-labeled PCs contain a certain amount of inactive (nonquenching) label (18). The 55% remaining PC was a mixture of the inactive label and the DMoPC or DOPC. The latter lipids were always the main PC component.
was similar for the wild type and mutant T domains, with the
(9, 22). Just as importantly, Fig. 1 shows that the 330:350 ratio
with results showing partial unfolding of T domain at low pH
increased contact of Trp to solution at low pH, in agreement
exposure of Trp to a more polar environment. This suggests
domain in solution. There is a conformational change resulting
sitivity at 330 nm relative to that at 350 nm (21).

wavelength shifts in the Trp fluorescence of wild type T domain and T domain
Trp mutants. Since the His6 tag had no effect on T domain behavior, it
was not removed in subsequent experiments.

RESULTS

Fluorescence Properties of Mutant and Wild Type T Domains in Solution—The behavior of mutants with a single Cys at
residues 322, 333, and 356 were studied to examine the struc-
ture of the T domain in model membranes. These mutants were
chosen because they are in the region containing TH8 and TH9,
two highly hydrophobic helices that have the capacity to form a
transmembrane structure (9–15).

We first examined whether the T domain mutants would
undergo the low pH-induced conformational change observed
in both whole toxin (20) and isolated T domain (9). This
conformational change is the critical step inducing membrane
penetration in cells (6, 7). The conformational change was
probed by measuring wavelength shifts in the Trp fluorescence
of the T domain. (T domain has two Trp residues, Trp206 in
helix 1 (TH1) and Trp281 in helix 5 (TH5)). Wavelength shifts
were detected by the ratio of Trp fluorescence emission inten-
sity at 330 nm relative to that at 350 nm (21).

Fig. 1 shows the effect of pH on the 330:350 ratio for T
domain in solution. There is a conformational change resulting
in a lower ratio (i.e. a red shift) below pH 5, indicative of
exposure of Trp to a more polar environment. This suggests
increased contact of Trp to solution at low pH, in agreement
with results showing partial unfolding of T domain at low pH
(9, 22). Just as importantly, Fig. 1 shows that the 330:350 ratio
was similar for the wild type and mutant T domains, with the

4 S. E. Malenbaum, R. J. Collier, and E. London, unpublished
observations.

FIG. 1. Effect of pH on Trp fluorescence of wild type T domain and unlabeled T domain mutants. Shifts in emission wavelength
were monitored by the ratio of emission at 330 nm to that at 350 nm. A decrease in this ratio is equivalent to a red shift in emission wave-
lengths. Samples contained wild type T domain (+), 332C (+), 335C
(●), 333C (○), and 356C (□). Other details are given under "Experimental Procedures."

low pH transition occurring at about pH 5 in all cases. This
shows that the introduction of a Cys has no significant effect on
the low pH-induced transition.

Fluorescence Properties of Bimane-labeled Mutant T Dom-
ains in Solution—To examine the effect of pH on the behavior
of the TH8/TH9 region, the experiment above was repeated
with bimane-labeled Cys mutants. Bimane was chosen because it
is a relatively small, uncharged, and fluorescent Cys-specific
reagent.

The emission properties of the T domain-attached bimane in
aqueous solution depended on its attachment site. Shifts in
bimane fluorescence were measured by the ratio of bimane
emission intensity at 440 nm relative to that at 480 nm. At
neutral pH, bimane-labeled 322C and 356C mutants had a
relatively low 440:480 ratio (i.e. were red-shifted), and labeled
333C had a much higher ratio (Fig. 2). This suggests that
bimane attached to 333C is in a relatively nonpolar, buried
location, consistent with the inability to label 333C without
first unfolding the T domain (see "Experimental Procedures")
and with both ESR studies (14) and the crystal structure of the
toxin.

The effect of pH on probe fluorescence was then examined.
As shown in Fig. 2, a conformational change was observed at
low pH with bimane-labeled 356C undergoing a blue shift and
labeled 333C undergoing a large red shift. These results show
that the TH8/TH9 region participates in the low pH-induced
change, in agreement with previous studies (9, 14). The pH
transition for labeled 333C occurs at a somewhat higher pH (by
1–1.5 pH units) than for the unlabeled protein. This may reflect
a destabilization of the folded, neutral pH conformation of the
T domain relative to the more unfolded low pH form by the
buried bimane label. Bimane-labeled 322C remained red-shifted at all pH values. This suggests it remains exposed to aqueous solution both at neutral and low pH.

**Fluorescent Properties of Model Membrane-inserted T Domain: Conformation Is Affected by Bilayer Structure**—T domain readily inserts into the bilayer of model membrane vesicles at low pH (9, 14, 15). The binding of the labeled T domain mutants to 30% PG, 70% PC (mol/mol) vesicles was tight, with concentrations of 100 μM lipid sufficient for complete binding as judged by fluorescence intensity changes upon lipid binding and centrifugation experiments (data not shown).

Our first experiments on T domain inserted into DOPG/DOPC vesicles indicated that the bimane-labeled 322C, 333C, and 356C all occupied shallow depths in the bilayer (see below). This was a surprise, because of the hydrophobic nature of TH8 and TH9. Therefore, we searched for conditions that would regulate the depth of T domain insertion. We recently found that bilayer width can control the transmembrane orientation of hydrophobic helices (23). Therefore, we examined whether bilayer width would affect the insertion of the T domain. This was first done by comparing fluorescence in DMoPC-containing vesicles relative to that in DOPC-containing vesicles. DMoPC has the same double bond position as DOPC but has 14 carbon acyl chains in place of the 18 carbon fatty acyl chains of DOPC. This decrease of 4 carbons results in about 7 Å thinner bilayers for the pure lipids (24, 25).

As shown in Fig. 3 and Table I, all three bimane-labeled mutants gave relatively red-shifted fluorescence in DOPG/DOPC vesicles. This suggests that in these vesicles all three residues are located in a relatively polar environment. However, bimane-labeled 333C and 356C underwent a distinct blue shift in DOPG/DMoPC vesicles relative to that in DOPG/DOPC, as judged both by λ<sub>max</sub> and the 440:480 ratio, although bimane-labeled 322C did not. This indicated that some conformational change occurred in the T domain in which residues 333 and 356, which are within the hydrophobic regions of helices TH8 and TH9, move to a more nonpolar location in DMoPC-containing vesicles. In other words, these results suggest there is deeper insertion of the T domain in DMoPC-containing vesicles than in DOPC-containing vesicles. The lack of a response in bimane-labeled 322C fluorescence to the change in lipid composition does not contradict this model, because residue 322, which is at the polar end of TH8, would not be expected to become buried in the membrane in any case.

**Fluorescent Properties of Model Membrane-inserted T Domain: Dependence on Bilayer Width**—To examine the correlation between bilayer width and conformation in more detail, the 440:480 and λ<sub>max</sub> ratio was examined for T domain inserted into vesicles containing PC with various acyl chain lengths (Fig. 4). This experiment showed that bimane-labeled 333C and 356C occupy more nonpolar locations (i.e. give blue-shifted fluorescence) when acyl chain length is less than 18 carbons. Similar effects were observed in the absence of DOPG (data not shown).

The effect of bilayer width was also examined by altering width in situ with decane. The addition of decane has been
shown to increase bilayer width in several studies (26, 27), and we found that it alters the transmembrane insertion of hydrophobic helices in the same manner as increasing acyl chain length (23). Fig. 5 shows that the addition of decane to DMoPC-containing vesicles partially or totally reverses the blue shift seen with bimane-labeled 333C and 356C without affecting the fluorescence of bimane-labeled 322C. This is consistent with the effect of bilayer width on T domain conformation described above. In addition, it demonstrates these conformations can be interconverted in situ. In other words, T domain inserted in DMoPC-containing vesicles was not permanently trapped in the “blue-shifted” (i.e., deeply inserted) conformation. This suggests that there is a dynamic equilibrium between the two conformations (also see below).

Fluorescent Properties of Model Membrane-inserted T Domain: Effect of Protein Concentration—Altering the concentration of T domain within the bilayer had an effect similar to that of changing bilayer width. As shown in Fig. 3 and Table I, when wild type T domain was added to the bimane-labeled protein incorporated into DOPG/DOPC vesicles, a blue shift was observed for bimane-labeled 333C and 356C, but not for labeled 322C. Fig. 6 shows that the addition of 5–10 μg of unlabeled T domain to 2.5 μg of bimane-labeled T domain was sufficient to obtain a maximal blue shift in fluorescence. These results suggest that there is an interaction between T domains that affects their conformation.

The different T domain conformations predominating at different protein concentrations are also in a dynamic equilibrium. This is shown by the fact that the subsequent addition of unlabeled T domain to membrane-inserted labeled T domain affected the conformation of the labeled protein. This would not have been observed if the labeled T domain had been permanently trapped in the “red-shifted” (i.e., shallow) conformation upon insertion.

The interaction between T domain molecules did not depend on the absolute protein concentration but rather on the concentration of T domain in the bilayer. This was shown by studies on bimane-labeled 356C in which lipid concentration was varied instead of protein concentration. Blue-shifted fluorescence was obtained at low lipid concentrations (data not shown). Thus, it is the protein:lipid ratio (i.e., the concentration of T domain in the bilayer) that determines the shift in emission wavelength.

There was no further blue shift of bimane-labeled 356C or 333C fluorescence seen when protein concentration was increased in DOPG/DMoPC vesicles (data not shown). This suggests that increasing protein concentration has no additional

| Bimane-labeled mutant | Parameter/Condition | \( \lambda_{\text{max}}^{a} \) | \( F_{440}/F_{480}^{c} \) |
|-----------------------|---------------------|-----------------|-----------------|
|                       | DOPC\(^{b}\) | DMoPC | DOPC + unlabeled T | DOPC | DMoPC | DOPC + unlabeled T |
| 322C                  | 470              | 471   | 469              | 0.44 ± 0.03 | 0.40 ± 0.03 | 0.43 ± 0.06 |
| 333C                  | 471              | 464   | 465              | 0.39 ± 0.04 | 0.66 ± 0.02 | 0.61 ± 0.11 |
| 356C                  | 469              | 462   | 462              | 0.46 ± 0.09 | 0.73 ± 0.04 | 0.76 ± 0.02 |

\(^{a}\) \( \lambda_{\text{max}} \) values were generally reproducible to within ±1 nm.

\(^{b}\) Model membranes were as follows: DOPC, DOPC-containing (30% DOPG, 70% DOPC (mol/mol)); DMoPC, DMoPC-containing (30% DOPG, 70% DMoPC); unlabeled T, unlabeled wild type T domain.

\(^{c}\) These values are the average and S.D. of 3–5 experiments.

Fig. 4. Effect of lipid acyl chain length on fluorescence of bimane-labeled T domain mutants. Samples contained T domain inserted into model membrane vesicles at pH 4.5. Left, \( \lambda_{\text{max}} \); right, ratio of emission intensity at 440 nm to that at 480 nm. Samples contained bimane-labeled 322C (+), 333C (■), and 356C (□).

![Image of graph showing effect of lipid acyl chain length on fluorescence of bimane-labeled T domain mutants.](image)
effect on the deeply inserted conformation present in DOPG/DMoPC vesicles.

Fluorescence Quenching by Spin-labeled Lipids: Assessing the Depth of Labeled Cys Residues—The experiments above show that residues 333 and 356 move into a more nonpolar environment in thin bilayers and at high T domain concentration in the bilayer. Fluorescence quenching by spin(nitroxide)-labeled lipid was measured to determine more directly whether these emission shifts actually reflect a change in membrane depth of these residues. To do this, the quenching of the labeled T domain mutants 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. When there are two populations, one deep and one shallow, the quenching by the deep and shallow nitroxide is stronger than by the medium depth nitroxide.

Fluorescence Quenching by Spin-labeled Lipids: Bimane Attached to 322C, 333C, and 356C Locates Shallowly in DOPC-containing Vesicles at Low T Domain Concentration—Table II illustrates the quenching of the labeled T domain mutants by lipids carrying a shallow (TempoPC), medium (5SLPC), or deep (12SLPC) nitroxide. A deep or shallow location of a fluorescent group is indicated when the quenching is strongest by the deep or shallow nitroxide, respectively. When there are two populations, one deep and one shallow, the quenching by the deep and shallow nitroxide is stronger than by the medium depth nitroxide.

Fluorescence Quenching by Spin-labeled Lipids: Deep Location of Bimane Attached to 333C and 356C Both in DMoPC-containing Vesicles and in DOPC-containing Vesicles at High T Domain Concentration—Table II also provides information on the location of residues 333 and 356 in DMoPC-containing bilayers and in DOPC-containing bilayers at high protein concentration. Both residues 333 and 356 are located more deeply under these conditions. This is shown by the increase in quenching by the deep 12SLPC nitroxide relative to that both by the medium 5SLPC and shallow TempoPC nitroxide (decrease in $F_{12SLPC}/F_{TempoPC}$; $\Delta < 0$). Since DMoPC and high protein concentration are also the conditions under which bimane-labeled 333C and 356C exhibit a blue shift, quenching confirms that the emission wavelength changes reflect deeper penetration of the T domain into the bilayer.5

Quenching also shows bimane attached to 333C and 356C must be present at more than one depth in DMoPC-containing vesicles and at high T domain concentration, since the quenching by the shallow and deep nitroxides is greater than that by the intermediate nitroxide. This indicates there must be coexisting shallow and deeply penetrating populations (Fig. 7).6

5 One concern we had in these experiments is that in the thinner DMoPC-containing bilayers quenching by 12SLPC could arise from 12SLPC molecules in both leaflets of the bilayer (18). This could increase 12SLPC quenching and make even a shallow residue appear deep. This possibility can be ruled out because 1) bimane-labeled 322C does not appear to be at a deep location in DMoPC-containing vesicles; 2) deep locations of the bimane-labeled 333C and 356C are also observed at high T domain concentrations, where this problem is not an issue; and 3) antibody binding confirms the deep location of labeled 333C and 356C.

6 Because of the presence of two populations at different depths, it is not useful to use the parallax analysis (18, 19) to determine average bimane depth.
Fluorescence Quenching By Spin-labeled Phospholipids: Bimane Attached to Residue 322 Remains Shallow under All Conditions—Table II also shows that, in contrast to the behavior of bimane-labeled 333C and 356C, bimane-labeled 322C remains at a shallow location in DMPC-containing bilayers and at high protein concentration. This is shown by the observation that the shallow TempoPC nitroxide gives the strongest quenching under all conditions. This result is in agreement with the lack of a blue shift for bimane-labeled 322C under any conditions.

Assessing T Domain Conformation by a Novel Antibody Binding Assay—As an additional approach to confirming the ability of the T domain to take on two different conformations, an antibody binding assay that takes advantage of the quenching of BODIPY fluorescence by anti-BODIPY antibodies was developed. Anti-BODIPY binding to BODIPY-labeled T domain mutants was used to assess the exposure of the labeled residues to external solution.

Table III shows the quenching of BODIPY-labeled T domain mutants by anti-BODIPY at pH 4.5 buffer. The results shown are the average of at least two experiments.

### DISCUSSION

The Two Different Conformations of Membrane-inserted T Domain—This study demonstrates that membrane-inserted T domain occurs in at least two conformations. In one conformation, residues in TH8 and TH9 have a shallow location, suggesting a form in which these helices are close to the surface. In the other form, the helices must be more deeply inserted into the bilayer (Fig. 7). One question to be resolved in future studies is whether the deeply inserted form is fully transmembrane.

Comparison with Previous Studies of T Domain Conformation—The existence of a conformation of the T domain in which TH8 and TH9 are close to the bilayer surface was not detected previously by ion conductivity or ESR studies (9–15). In the former case, this is not unexpected because it is only the pore-forming transmembrane population that is detected. On the other hand T domain spin-labeled mutants, ESR, which has the potential to detect both shallow and deeply inserted species, showed that the structure of TH8 and TH9 was more consistent with a transmembrane structure. Based on the lipid and protein concentrations used in the ESR studies, the fluorescence results suggest that a mixture of the shallow and deeply penetrating conformations could have been present. It is possible that subtle differences in experimental conditions, differences between labeling groups, or different sensitivity to the presence

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**TABLE II**

| Bimane-labeled mutant/condition | \( F_{12SLPC}/F_0 \) | \( F_{SLPC}/F_0 \) | \( F_{TempoPC}/F_0 \) | \( F_{12SLPC}/F_{TempoPC} \) | \( \Delta \) |
|---------------------------------|-----------------|-----------------|------------------|-------------------|------|
| 322CdOPC                        | 0.84 ± 0.04     | 0.84 ± 0.05     | 0.78 ± 0.02      | 1.08 ± 0.05       | 0    |
| 322cDMoPC                       | 0.82 ± 0.03     | 0.80 ± 0.04     | 0.72 ± 0.05      | 1.15 ± 0.08       | +0.07|
| 322cDOPC + unlabeled T          | 0.94             | 0.90             | 0.83             | 1.12              | +0.04|
| 333cDOPC                        | 0.75 ± 0.04     | 0.72 ± 0.03     | 0.64 ± 0.03      | 1.18 ± 0.03       | 0    |
| 333cDMoPC                       | 0.60 ± 0.02     | 0.70 ± 0.03     | 0.62 ± 0.02      | 0.97 ± 0.04       | −0.19|
| 333cDOPC + unlabeled T          | 0.74 ± 0.04     | 0.80 ± 0.01     | 0.74 ± 0.02      | 0.99 ± 0.04       | −0.17|
| 356cDOPC                        | 0.72 ± 0.04     | 0.67 ± 0.06     | 0.63 ± 0.03      | 1.15 ± 0.06       | 0    |
| 356cDMoPC                       | 0.63 ± 0.06     | 0.74 ± 0.08     | 0.67 ± 0.01      | 0.94 ± 0.05       | −0.21|
| 356cDOPC + unlabeled T          | 0.73 ± 0.05     | 0.72 ± 0.14     | 0.71 ± 0.03      | 1.03 ± 0.02       | −0.12|

* \( F_0 \) is the fluorescence in model membrane vesicles lacking spin-labeled lipid. \( F_{12SLPC}, F_{SLPC}, \) and \( F_{TempoPC} \) are fluorescence intensities in the vesicles containing spin-labeled lipids. The ratio \( F/F_0 \) gives the fraction of fluorescence remaining unquenched by the spin labels. The values shown are generally the average and S.D. of 3–5 experiments.

**TABLE III**

| BODIPY-labeled mutant | Exposure (percentage of maximum quenching) |
|-----------------------|------------------------------------------|
|                       | %                                       |
| 322C                  | 66                                       |
| 333C                  | 67                                       |
| 356C                  | 59                                       |
| DOPC + unlabeled T    | 68                                       |

* Values shown are the percentage of quenching relative to that obtained for free BODIPY-iodoacetamide probe (74%) in the same pH 4.5 buffer. The results shown are the average of at least two experiments.

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This conclusion is supported by the observation that in aqueous solution maximal antibody binding to T domain in the native conformation exhibits a similar level of quenching to that observed in DOPC-containing vesicles (not shown).

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**FIG. 7.** Schematic diagram showing the difference in the two conformations of the T domain. The relative positions of residues 322, 333, and 356 are indicated by the closed circles. Helices TH8 and TH9 are indicated by rectangles.

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[Diagram showing the difference in the two conformations of the T domain.]

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This study demonstrates that membrane-inserted T domain occurs in at least two conformations. In one conformation, residues in TH8 and TH9 have a shallow location, suggesting a form in which these helices are close to the surface. In the other form, the helices must be more deeply inserted into the bilayer (Fig. 7). One question to be resolved in future studies is whether the deeply inserted form is fully transmembrane.
of mixtures explains the differences between the ESR and fluorescence results.

*T Domain Protein-Protein Interactions*—How does high *T* domain concentration promote deep insertion? The most likely possibility is that at high *T* domain concentration the *T* domain forms oligomers within the bilayer. Oligomers could allow for deep insertion by burying polar residues within their interior, *i.e.* away from the lipid bilayer. Oligomerization has already been identified as a key step in membrane insertion by anthrax toxin and α-hemolysin (28, 29). In the case of α-hemolysin, there is a shallowly inserted form, which is believed to be converted to the transmembrane state upon formation of an oligomeric structure in the membrane (30). Therefore, it would not be surprising if oligomeric interactions were important for deep diptheria toxin insertion as well. Some evidence for oligomeric interactions between diptheria toxin molecules has been previously obtained from studies of pore formation and other properties (31–33). In preliminary studies, we have more directly detected oligomer formation in membrane-inserted whole toxin. However, the nature of the oligomeric species is not yet clear. For example, whether there is a specific oligomeric stoichiometry is unknown. The presence of co-existing deep and shallow populations at high *T* domain concentrations brings up the unusual possibility of the formation of an oligomer in which different *T* domains have different positions in the bilayer. Alternately, there may simply be a mixture of separate shallow and deep populations.

Effect of Bilayer Width on Insertion—This study also demonstrated bilayer width could influence the degree of membrane penetration. Specifically, deep insertion was observed in thin DMPC-containing bilayers. One possible explanation is that oligomerization of the *T* domain is promoted in thin bilayers, so that oligomers are present even at dilute concentrations in such bilayers. This is supported by the observation that the deep insertion inferred from bimane fluorescence in DMPC-containing bilayers is concentration-independent. Alternately, a deeply inserted monomeric structure may form. Transmembrane insertion may simply be promoted by the fact that in a thinner bilayer fewer polar groups must be buried within a hydrophobic milieu. In any case, these results suggest that altering bilayer thickness may be a useful tool for manipulating membrane protein insertion.

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