Identifying Transmembrane States and Defining the Membrane Insertion Boundaries of Hydrophobic Helices in Membrane-inserted Diphtheria Toxin T Domain*

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The membrane topography of proteins that convert between soluble and membrane-inserted states has proven a challenging problem. In particular, it has been difficult to define both whether a transmembrane orientation is achieved and what are the boundaries of membrane-inserted segments. In this report the fluorescence of bimane-labeled Cys residues and the binding of anti-BODIPY antibodies to BODIPY-labeled Cys residues are combined to define these features for helices TH8 and TH9 of the T domain of diphtheria toxin. Using a series of labeled residues the topography of these helices was examined in both conformations of membrane-inserted T domain identified previously (Wang, Y., Malenbaum, S. E., Kachel, K., Zhan, H., Collier, R. J., and London, E. (1997) J. Biol. Chem. 272, 25091–25098). In the shallowly inserted conformation these helices are found to be aligned close to the cis surface of the bilayer all along their sequences. In contrast, in the more deeply inserted conformation most TH8 and TH9 residues examined located in a non-polar environment, with the boundaries of the membrane-inserted sequences close to residues 324 and 372–374 on the cis (insertion) side of the bilayer. It was also found that residues 348 and 349, which are in the loop connecting TH8 and TH9, reached the opposite trans side of the bilayer, but did not protrude fully into the aqueous environment. These boundaries suggest the membrane-inserted segments of TH8 and TH9 form transmembrane helices about 25 residues in length, and suggest that they are connected by a tight turn. It is concluded that this combination of fluorescent techniques can be combined to obtain transmembrane helix topography.

Diphtheria toxin is secreted by Corynebacterium diphtheriae. The toxin can be split into two chains, A (Mr 37 kDa) and B (Mr 21 kDa) joined by a disulfide bond (1). The crystal structure shows diphtheria toxin 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. Subsequent to endocytosis, the toxin reaches endosomes and membrane penetration occurs (6). The low pH within the endosomal lumen induces a partial unfolding of the toxin, resulting in exposure of the hydrophobic regions and translocation of the A chain of the toxin into the cytoplasm (6). In the cytoplasm, the A chain catalyzes the transfer of ADP-riboyl group of NAD+ to elongation factor 2, inactivating protein synthesis.

The T domain is composed basically of α-helices, several of which are hydrophobic and are likely to play a critical role in membrane insertion and translocation (2, 7). Determining whether such helical hydrophobic segments span the bilayer has been an especially difficult problem for those proteins that, like the T domain, convert between soluble and membrane-inserted forms. For example, despite much study the boundaries of the inserted sequence and the orientation of the hydrophobic tail of cytochrome b5 in its loosely and tightly bound states has proven complex (8, 9). In the case of some colicin, channel domains determining the conditions under which the two most hydrophobic helices are transmembrane (umbrella model) or non-transmembrane (penknife model) has also been a difficult question (10–12).

For the T domain of diphtheria toxin recent studies have concentrated on the two most hydrophobic helices, TH8 and TH9, because they are most likely to form a transmembrane structure upon membrane insertion (13–20). Studies on pore formation by T domain mutants are consistent with a model in which in the loop between TH8 and TH9 reaches the trans side of the membrane in the pore-forming state (14–18). (The trans side is the membrane surface opposite that (cis) into which the toxin initially inserts.) However, the details of the orientation and structure of these helices are not yet clear. Studies using electron spin resonance (ESR) have suggested a transmembrane structure for TH9 with a defined pore facing side, but did not define the precise helix boundary on the trans side of the bilayer (see “Discussion”) (19, 20). In addition, pore formation studies measuring the effects of reaction with aqueous methanethiosulfonate reagents have been interpreted in terms of a flexible non-helical structure for both TH8 and TH9 (21). Complicating matters, our recent fluorescence studies have shown that the T domain can exist in two distinct conformations, one of which penetrates the bilayer to a much greater extent than the other (22).

Therefore, additional methods to identify the membrane-inserting boundaries and to establish the degree of transmembrane orientation of TH8 and TH9, and analogous sequences in other proteins, are desirable. In this report the locations of a series of T domain residues labeled with bimane and BODIPY fluorophores have been evaluated by fluorescence and fluorescence quenching-detected antibody binding. The results identify the more deeply inserted conformation of the T domain as transmembrane, and place narrow limits on the boundaries of the membrane-inserted sequence.
**EXPERIMENTAL PROCEDURES**

**Materials**—Dioleoyl PC (DOPC), 1 dimyristoyl-PC (DMoPC), and dioleoyl-PC (DOPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Lipid concentrations were determined by dry weight. Rabbit anti-BODIPY-FL IgG, N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-yl)methyliodoacetamide (BODIPY-FL C1, IA, BODIPY-iodoacetamide), and monochlorobimane were purchased from Molecular Probes (Eugene, OR). All other chemicals were reagent grade.

Preparation of T domain from *E. coli* strains expressing T domain mutants with single Cys substitutions was performed similarly to the methods described previously (22). Resequencing of the mutants detected only one unexpected mutation, a T267I substitution in the mutant carrying a Cys on residue 361. A Talon Metal affinity resin was used in place of a nickel-charged His binding for purification (CLONTECH, Palo Alto, CA). Generally, 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, 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 0.5-ml aliquots of elution buffer (1 × imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 8). The T domain tended to elute in the last wash buffer and the 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 fast protein liquid chromatography 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 ml Tris-Cl, pH 8. The T domain eluted at about 250 mM NaCl. The purified fractions (detected by SDS-gel electrophoresis) were combined and stored at 4 °C. The hexa-His tag at the N-terminal was not removed (22). Generally, final purity appeared to be over 95% as judged by SDS-gel electrophoresis. T domain concentration was determined from the absorbance at 280 nm using ε = 18,200 M⁻¹ cm⁻¹, and converted to micrograms using an approximate molecular weight of 20,000.

**Fluorescence Labeling of T Domain**—Those T domain mutants which leaked efficiently without unfolding were labeled with BODIPY-iodoacetamide and monochlorobimane as described previously (22), except that for bimane labeling 3.8 μl of 8 mM monochlorobimane dissolved in ethanol was added to the T domain sample and the incubation at room temperature was 45–60 min before dialysis. In all cases, it was assumed that there was full recovery of protein (22).

**Fluorescence Labeling of T Domain Under Denaturing Conditions**—For several mutants the level of labeling was very low unless they were unfolded during the labeling reaction. These mutants (328, 331, 333, 349, 361, 366, 368, and 378) were labeled under denaturing conditions and then refolded as described previously (22). A small aliquot of solution containing 50 μg of mutant T domain was combined with an equal volume of urea, yielding a volume of 50–200 μl. To this solution, 3.8 μl of 8 mM monochlorobimane in ethanol or 6.4 μl of 1.95 mM BODIPY-iodoacetamide in dimethyl sulfoxide was added and then mixed with a micro-stir bar for 1 h. To induce refolding of the T domain, samples were then diluted rapidly while mixing with 10 ml Tris, 140 mM NaCl, pH 8, to a final volume where the urea concentration was between 0.2 and 0.4 M (22). Both residual urea and excess fluorescent label were removed by dialysis, as described previously (22). The Trp emission of the labeled protein (with or without the denaturation step) was generally between 334 and 338 nm, which was close to the value observed with the native wild type T domain. In all labeling experiments wild type T domain was labeled (using the same protocol as for the mutants) in parallel to the mutants. The degree of labeling of wild type T domain was labeled (using the same protocol as for the mutants) in parallel to the mutants. The degree of labeling of wild type T domain was determined from the absorbance at 280 nm using ε = 18,200 M⁻¹ cm⁻¹, and converted to micrograms using an approximate molecular weight of 20,000.

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**Results**

**Attaching the Structure of Membrane-inserted T Domain**—Fluorescence assays were used to evaluate the location of bimane residues introduced into the TH8/TH9 helical hairpin region of the T domain. In the first, the emission wavelength of bimane-labeled residues was measured. This parameter is sensitive to exposure to aqueous solution. In the second, the accessibility of BODIPY-labeled Cys residues to labeling by anti-BODIPY antibodies was determined from the quenching of BODIPY fluorescence upon antibody addition. Anti-BODIPY binding can only occur when a residue is exposed to the external aqueous solution (22).

**Anti-BODIPY Bion and T Domain in the P and TM Conformations**—As shown in Fig. 1 there is a marked dependence of antibody binding to BODIPY-labeled residues upon both residue position and T domain conformation. In the DOPC-containing bilayers T domain inserts into bilayers with both of these lipid mixtures, but in a less deeply inserted conformation in vesicles formed by DOPC than in the thinner bilayers formed by DMoPC (22). The shallower conformation will be denoted as the P (partially inserted) conformation and the deeper conformation the TM (transmembrane) conformation for reasons that will become clear.

2 That BODIPY groups exposed at the membrane surface can bind to antibody is confirmed by the efficient antibody-induced quenching of a BODIPY group attached to the amino group of phosphatidyethanolamine (BODIPY FL C1, DHPE, Avanti Polar Lipids) (data not shown). In this liquid, the BODIPY group locates in the polar head group region of the bilayer, about 21 Å from the bimane label. That antibody binding requires a residue to be exposed on the external surface of the membrane is confirmed by leakage studies showing that the T domain does not form pores large enough to allow the antibodies to pass through the bilayer (J. Sharpe and E. London, unpublished observations).

1 The abbreviations used are: DOPC, di-C18:1,4-dihexadecanoylphosphatidylcholine; DOPG, di-C16:1,4-dihexadecanoylphosphatidylglycerol; PC, phosphatidylcholine; TM, transmembrane.
The position of the Cys residue in the T domain mutant sequence is given on the X axis. Samples contained T domain inserted into small unilamellar vesicles containing 30% DOPG and 70% DMoPC or DOPC at pH 4.5. The average of duplicate samples is shown. Individual samples generally exhibited quenching within 3–4% of these values.

This model predicts that upon switching from DOPC-containing vesicles to DMoPC-containing vesicles, not only would those non-helix flanking residues within TH8 and TH9 that are buried in DOPC-containing vesicles would lose half their reactivity. In contrast, consistent loss of half of the reactivity would not be predicted by a model in which a single conformation is present in the DMoPC-containing samples. Therefore, the observation that the former prediction is borne out by the behavior of residues 361 and 368, which are the relatively unreactive with antibody in the DOPC-containing vesicles, yet like the more reactive residues lose about half their reactivity in DMoPC-containing vesicles, further supports the two-conformation model.

Thus, the loss of antibody reactivity in DMoPC-containing vesicles relative to DOPC-containing vesicles identifies residues that become inaccessible to antibody in the TM state. The fact that this loss is seen with all but the residues at the N-terminal end of TH8 and C-terminal end of TH9 implies that the insertion of the TH8/TH9 region is oriented with only the N-terminal flank of TH8 and C-terminal flank of TH9 exposed to the external (cis) solution in the TM state. Even the TL5 loop sequence (residues 347–351), which contain Asp and Glu residues, is inaccessible to antibody in solution in DMoPC-containing vesicles. This pattern is most easily explained by a structure in which TH8 and TH9 form a transmembrane helical segment on the cis side of the bilayer is close to residue 324 on TH8 and 372 on TH9 (Fig. 3, top).

**Bimane Fluorescence of Labeled T Domain in the P and TM Conformations**—To confirm and amplify the conclusions obtained with anti-BODIPY antibodies, the fluorescence emission of bimane-labeled Cys mutants was monitored. As shown in Table I, residues were assigned to three classes, those exposed to a non-polar environment (which exhibited a blue shifted wavelength of maximum fluorescence emission), those exposed to a polar environment (which exhibited a red shifted fluorescence maximum), and those exposed to an intermediate environment (which gave an intermediate wavelength shift).

3 In the crystal structure toxin residues 352–357 form a distorted helical region. On the assumption that a more ordered helical structure is likely to form within the bilayer, we have included these residues in TH9.
In contrast, in DMOPC-containing vesicles most residues exhibited a significantly more blue-shifted fluorescence, with an emission maximum shifted to below 465 nm. This shows that the conformational change detected by anti-BODIPY binding can also be detected by bimane emission.

To analyze the results in the DMOPC-containing vesicles in more detail, it was necessary to determine the emission maximum of the subpopulation of T domain molecules that are in the TM conformation, i.e., to correct for the presence of the T domain molecules that are not deeply buried. We did this roughly by utilizing the estimate from the anti-BODIPY results that 50% of the molecules in the DMOPC-containing vesicles are in the P conformation (or in a similar conformation), and estimating that the shift between the emission maximum in the DMOPC-containing vesicles and the DOPC vesicles would be about double if the T domain DMOPC-containing vesicles were 100% in the TM conformation. This rough approximation should be adequate because only small shifts in wavelength are being considered, and the overall intensity difference of bimane-labeled residues in DOPC and DMOPC is not large (not shown).4

As shown in Table I, the estimated emission maximum values for the pure TM state show that the residues with the most striking red shifts were mainly in the regions flanking the N terminus of TH8 and C terminus of TH9, namely 322, 324, and 378. This indicates that these residues are fully exposed to aqueous solution in the TM conformation, in good agreement with the anti-BODIPY results. All other residues exhibited more blue-shifted fluorescence, with the exception of residue 338. Its anomalous behavior is considered under "Discussion." Several residues showed an intermediate red-shifted fluorescence.

![Table I](image)

| Conformation (conditions), labeled Cys residue | Emission maximum (nm) |
|-----------------------------------------------|-----------------------|
| Mixed deep (DMoPC-containing vesicles) | P conformation (DOPC-containing vesicles) | TM conformation estimated |
| 322 | 469S* | 470 S | 468 S |
| 324 | 470 | 464 M | 475 S |
| 328 | 465 | 466 M | 464 M |
| 331 | 460 | 468 S | 462 d |
| 333 | 463 | 471 S | 455 d |
| 338 | 473 | 474 S | 474 S |
| 341 | 469 | 473 S | 463 d |
| 344 | 466 | 471 S | 461 d |
| 347 | 459 | 468 S | 451 d |
| 348 | 470 | 474 S | 466 M |
| 349 | 467 | 468 S | 466 M |
| 351 | 463 | 471 S | 455 d |
| 352 | 464 | 471 S | 457 d |
| 353 | 466 | 470 S | 462 d |
| 354 | 461 | 469 S | 453 d |
| 355 | 461 | 469 S | 453 d |
| 358 | 461 | 470 S | 452 d |
| 361 | 461 | 463 d | 459 d |
| 366 | 466 | 471 S | 461 d |
| 368 | 466 | 467 S | 465 M |
| 371 | 464 | 468 S | 460 d |
| 374 | 467 | 468 S | 466 M |
| 378 | 470 | 470 S | 470 S |

* Results shown are the average of duplicate samples. Wavelength maxima were reproducible to within ±1 nm, except for residue 338 in DOPC-containing vesicles where the range was ±2 nm. Data for residues 322, 333, and 356 from Ref. 22.

4 It should be noted that the details of the transmembrane conformation obtained by correcting for a non-transmembrane subpopulation would only be altered slightly if the correction was omitted and the raw anti-BODIPY quenching and bimane emission maxima were used to evaluate topography.

For almost all residues the wavelength of maximum emission was highly red shifted in DOPC-containing vesicles, suggesting that almost every residue examined in TH8 and TH9 was fully exposed to a polar environment. This indicates, in excellent agreement with the anti-BODIPY binding results, that in the P conformation the TH8/TH9 helix pair lies close to the cis surface of the bilayer. It should be noted that the large blue shift of residue 361 in the P conformation (Table I), which is an exception to this pattern, is consistent with the inaccessibility of this residue to antibody binding in DOPC-containing vesicles (Fig. 1).
values were reproducible to within ±1 nm.

| Labeled residue | Experimental | TM (estimated) |
|-----------------|--------------|----------------|
| 322             | 469          | 468 S          |
| 324             | 463          | 461 d          |
| 328             | 466          | 466 M          |
| 331             | 464          | 460 d          |
| 333             | 463          | 455 d          |
| 338             | 474          | 474 S          |
| 341             | 469          | 463 d          |
| 344             | 466          | 461 d          |
| 347             | 466          | 464 M          |
| 348             | 471          | 468 S          |
| 349             | 466          | 466 M          |
| 351             | 465          | 462 d          |
| 352             | 463          | 455 d          |
| 353             | 465          | 460 d          |
| 354             | 461          | 453 d          |
| 356             | 461          | 453 d          |
| 358             | 463          | 456 d          |
| 361             | 459          | 455 d          |
| 366             | 466          | 461 d          |
| 368             | 464          | 461 d          |
| 371             | 464          | 460 d          |
| 374             | 467          | 464 M          |
| 378             | 470          | 470 S          |

The behavior is consistent with an environment of intermediate polarity, e.g., in the polar head group region of the bilayer, rather than the more non-polar acyl chain region. Such residues fall into two groups. One set is residues 328, 368, and 374. A location of these residues in the polar head group region of the bilayer is consistent with their adjacent position to the fully exposed residues in the amino acid sequence of TH8 and TH9.

The other residues showing intermediate red shift fluorescence are 348 and 349. These residues are within the TL5 loop, which has been predicted to reach the trans side of the bilayer upon transmembrane insertion. Their intermediate red-shifted fluorescence suggest they reach the polar head group region of the trans bilayer face (Fig. 3), as predicted for a transmembrane conformation (see "Discussion").

In previous studies, we also established that the TM conformation could form in DOPC-containing vesicles at high T domain concentration. Table II shows that the bimane shifts under this condition are very similar to those in DMPC-containing vesicles, confirming that the conformation under these two conditions are similar. The main difference is that the membrane insertion boundaries on the helices shift slightly. At high concentrations in DOPC-containing vesicles the TL5 loop residues may reach slightly closer to the aqueous solution on the trans side of the bilayer, as judged by the larger red shift for residue 347 and 348; while on the cis side of the bilayer residues at the insertion boundaries of the N terminus of TH8 (i.e. 324) and C terminus of TH9 (i.e. 368) become more deeply buried, as shown by larger blue shifts (see Fig. 3). Reduced anti-BODIPY antibody binding was also observed for membrane-buried residues at higher T domain concentrations, but was variable due to interference from light scattering, which was stronger than at low T domain concentrations.

**DISCUSSION**

In the TM Conformation the TH8/TH9 Hairpin is Transmembraneous and Shows Oriented Insertion—The problem of refining the boundaries of membrane-inserted segments obtained from amino acid sequence data has proven difficult. As noted in the Introduction, unambiguous information has been especially difficult to obtain for proteins that switch between soluble and membrane-inserted state. This study demonstrates that the orientation and approximate boundaries of membrane-inserted segments can be identified by combining fluorescence methods, although it should be cautioned that fluorescence emission wavelength can be sensitive to solvent relaxation effects and polarization artifacts not considered here.

We previously speculated that the more deeply inserted conformation of the T domain was transmembraneous (22). The observations in this report confirm this model, and indicate that in the transmembrane conformation there is oriented insertion of the TH8/TH9 region such that the N-terminal end of TH8 and C-terminal end of TH9 remain on the cis surface of the bilayer. They also show the residues between 324 and 374 are not immersed in the external aqueous solution, and that 348 and 349 reach to the boundary of the trans side of the bilayer. In summary, in the TM conformation the TH8/TH9 helix pair is transmembraneous and oriented such that the loop connecting TH8/TH9 is on the trans face of the bilayer when the T domain is added externally to vesicles. The relatively unidirectional orientation of the membrane-inserted T domain contrasts with the mixed orientations we previously observed for membrane-inserted whole diphtheria toxin (26).

The Boundaries of Membrane-inserted Segments in The TM Conformation and Implications for the Insertion Process—The approximate boundaries of the bilayer found by fluorescence are in good agreement with the crystal structure and amino acid sequences of TH8 and TH9 and the surrounding residues. In the soluble state helix 8 spans residues 326–346 and helix 9 (if a distorted region from 352–357 is included) residues 352–376. These sequences are primarily composed of hydrophobic residues located in stretches between residues 328–348 and 353–371. The non-polar region of TH8 starts at residue 327, and the non-polar stretch of TH9 ends at residue 371. This compares favorably to the estimate from fluorescence that the insertion boundaries on the cis face of the bilayer are close to residue 324 flanking TH8 and 372–374 for TH9.

The boundaries of the membrane inserting sequences on the cis side of the bilayer also set limits on the location of the residues on the opposite (trans) end of TH8 and TH9 in the transmembrane state. For TH8 and TH9 to span the hydrocarbon portion and both polar regions of the bilayer would require about 25–30 residues. Therefore, if TH8 and TH9 form a helical hairpin, it would be unlikely for the residues in the TL5 loop (e.g. 348 and 349), which are only about 25 residues from the cis insertion boundary to fully reach the aqueous solution on the trans side of the bilayer, especially if TH8 and TH9 are tilted with respect to the bilayer to a significant degree. Instead, they would be expected to penetrate into the trans polar head group region (Fig. 3), as the bimane fluorescence emission results indicate. Thus, combining the bimane and BODIPY results, we conclude that in the TM state helices TH8 and TH9 are just barely transmembraneous, with a tight TL5 loop.
reaching the polar head group region on the trans side of the bilayer.

A structure in which there is a tight loop connecting TH8 and TH9 which does not protrude significantly from the bilayer is reminiscent of the situation in the α-hemolysin of Staphylococcus aureus and protective antigen subunit of anthrax toxin. In both of these toxins the transmembrane segment is comprised of a β-strand hairpin connected by a tight loop that just reaches the trans side of the bilayer (27, 28). It may be that such minimal transmembrane protrusion is a common feature of toxins. Such an arrangement would reduce the energetic cost of transmembrane insertion by reducing the number of polar residues that must be translocated across the bilayer to a minimum.

The anomalous behavior seen for residues 338 and 361 should also be noted. We assume that the behavior of residue 338, which is in the non-polar portion of TH8 yet appears to be close to the cis surface of the bilayer as judged by fluorescence, represents conformational perturbation originating from the substitution of Val by a Cys labeled with polar fluorophores (see below). A polar insertion at this residue may be especially perturbing because it is also adjacent to two polar Ser residues in the center of TH8, and so may render the center of TH8 sufficiently hydrophilic to disrupt transmembrane insertion.

Residue 361 is anomalous in that it appears to be buried in the P conformation. This may reflect its location adjacent to Glu-362. The tendency of Glu-362 to locate at the surface may overcome the polarity of the bimane and BODIPY probes attached at 361 to do so, forcing them to orient more deeply. Whatever the explanation of the behavior of residues 338 and 361, perturbations due to mutagenesis and labeling are always a concern in studies of the type undertaken here, and their behavior illustrates the importance of only formulating models for insertion from a series of residues, rather than relying on the behavior of a single residue.

**The Topography of TH8 and TH9 in the P Conformation—**

The uniform exposure of residues in the P conformation to the cis side of the membrane suggests in this conformation the T domain only partially penetrates the lipid bilayer. Since the TH8/TH9 region is helical it would have been predicted that in the P state the emission maximum of bimane fluorescence should have shown a dependence on position with a helical (3.6 residue) repeat, i.e. such that residues on the lipid facing side of the helix would have given more blue-shifted fluorescence than on the solution facing side (29). Instead a uniform red-shifted fluorescence was observed. This may arise because bimane is sufficiently polar to twist the helix in the P state so that it can face aqueous solution independent of the position the native residues would occupy. In support of this hypothesis, we have found that TH9 in the P conformation appears more deeply buried in the bilayer when probed with Trp substitutions than found here with the more polar bimane probe. 6,7

The uniform reactivity of BODIPY-labeled residues in the P state may reflect behavior similar to bimane. Studies of BODIPY-labeled lipids demonstrate that the BODIPY group is sufficiently polar to preferentially locate at the membrane surface even when attached to the end of fatty acyl chains (23). Furthermore, even if the BODIPY group usually faces aqueous solution for only a short period of time it could be trapped in the solution facing orientation by antibody binding.

**Comparison to Previous Studies—** As noted above, based on the crystal structure and hydrophobicity of TH8 and TH9, a transmembrane orientation and boundaries similar to those identified in this report were predicted or implied (2, 17, 30). However, experimental data has been more ambiguous. Studies of the structure of toxin pore formation have been able to identify whether a residue influencing the pore formed by the T domain is closer to the cis or trans side of the bilayer by manipulation of cis and trans pH (17, 31). However, a systematic attempt to estimate helix topography by examining the effect of chemical modification on pore properties instead fit a flexible, non-helical model for TH8 and TH9, at least in the open pore state (21). (This should be contrasted with the excellent fit to a “classical” β-sheet profile found for the membrane inserted segment of protective antigen of anthrax toxin determined by the same chemical modification approaches (27).)

A non-helical model is difficult to rationalize as representing the predominant conformation of TH8 and TH9 in terms of the other data described above and the results of this study. In addition, CD suggests the T domain remains highly helical when membrane inserted. 8 Perhaps the reactivity profile represents that of the P conformation, or a minor transmembrane state in which residue exposure is different from that in the predominant transmembrane state.

Another approach to TH9 topography has been ESR which, in addition to identifying lipid facing and polar facing sides of TH9, has defined a boundary for the C-terminal side of TH9 that is close to that found in this study. However, as presently used, ESR can only give an estimate of depth at every 3–4 residues (residues that face lipid) and does not distinguish between cis and trans facing residues.

In any case, all of these techniques represent powerful approaches to complex questions of membrane protein topography. It appears that for a complete topographic model several approaches will often need to be combined. The special advantages of the fluorescent methods introduced in this report make them powerful approaches for such studies.

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Diphtheria Toxin Helix Insertion

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