Topography of Helices 5–7 in Membrane-inserted Diphtheria Toxin T Domain

IDENTIFICATION AND INSERTION BOUNDARIES OF TWO HYDROPHOBIC SEQUENCES THAT DO NOT FORM A STABLE TRANSMEMBRANE HAIRPIN

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The T domain of diphtheria toxin undergoes a low pH-induced conformational change that allows it to penetrate cell membranes. T domain hydrophobic helices 8 and 9 can adopt two conformations, one close to the membrane surface (P state) and a second in which they apparently form a transmembrane hairpin (TM state). We have now studied T domain helices 5–7, a second cluster of hydrophobic helices, using Cys-scanning mutagenesis. After fluorescently labeling a series of Cys residues, penetration into a non-polar environment, accessibility to externally added antibodies, and relative depth in the bilayer were monitored. It was found that helices 5–7 insert shallowly in the P state and deeply in the TM state. Thus, the conformational changes in helices 5–7 are both similar and somehow linked to those in helices 8 and 9. The boundaries of deeply inserting sequences were also identified. One deeply inserted segment was found to span residues 270 to 290, which overlaps helix 5, and a second spanned residues 300 to 320, which includes most of helix 6 and all of helix 7. This indicates that helices 6 and 7 form a continuous hydrophobic segment despite their separation by a Pro-containing kink. Additionally, it is found that in the TM state some residues in the hydrophilic loop between helices 5 and 6 become more highly exposed than they are in the P state. Their exposure to external solution in the TM state indicates that helices 5–7 do not form a stable transmembrane hairpin. However, helix 5 and/or helices 6 plus 7 could form transmembrane structures that are in equilibrium with non-transmembrane states, or be kinetically prevented from forming a transmembrane structure. How helices 5–7 might influence the mechanism by which the T domain aids translocation of the diphtheria toxin A chain across membranes is discussed.

Diphtheria toxin (DT)† is a protein toxin secreted by the bacterium Corynebacterium diphtheriae. The mature toxin (58 kDa) consists of two polypeptide chains, A (21 kDa) and B (37 kDa), which are joined by a single disulfide bond. Solution of the crystal structure at neutral pH revealed that the toxin consists of three distinct domains. The A chain is identical to the catalytic (C) domain of the protein while the B chain consists of two domains, the receptor-binding domain (R) and the transmembrane (T) domain (2–5). Upon entering endosomes, via receptor-mediated endocytosis, a conformational change triggered by the low pH of the endosomal lumen renders the toxin hydrophobic. This results in membrane penetration by the toxin and subsequent translocation of the A chain into the cytosol. Once in the cytosol, the A chain catalyzes the ADP-ribosylation of the diphthamide residue of elongation factor 2, which shuts down protein synthesis and leads to cell death (6).

The details of the mechanisms by which protein toxins such as DT (or for that matter any proteins) translocate across membranes remain elusive. One reason is that toxins with helical membrane-inserted segments are challenging targets for topography studies. This arises from the fact that such proteins membrane insert only under a limited set of conditions, and can have membrane-inserted segments that are not as hydrophobic as those in normal transmembrane proteins. Nevertheless, the importance of understanding protein translocation and toxin action justifies detailed analysis of toxin topography. To date, topography studies have concentrated on the largely α-helical T domain, which contains several hydrophobic helices that have been suggested to play a crucial role in both membrane insertion and translocation (2, 7). The topography of these helices in the membrane-inserted state should shed light on the function of the T domain in translocation. Initial studies focused on the large, hydrophobic C-terminal helices 8 and 9 (TH8 and TH9). There has been evidence from a wide variety of techniques, such as ESR, proteolysis, and pore formation in planar lipid bilayers, suggesting that these helices form a stable transmembrane hairpin at low pH (8–17). We performed a series of detailed topographical studies on the isolated T domain in which it was demonstrated helices 8 and 9 can exist in one of two distinct conformations. In one, they lay close to the membrane surface (partially inserted (P) state), and in the second they insert more deeply (TM state) (18, 19). Whether the P or TM state predominates depends on a number of factors, including bilayer width, bilayer curvature, and T domain concentration (18–20). The insertion boundaries for deeply inserted helices 8 and 9 were defined and were consistent with a transmembrane helical hairpin joined by a tight turn (19). More recently, we found that helices 8 and 9 can be converted from the P to TM conformation upon interaction with many proteins that, like the A chain, adopt a molten globule-like state at low pH (20). This may suggest that the T domain possesses a chaperone-like quality which it uses to recognize...
the partially unfolded A chain, and which promotes A chain translocation (20).

The behavior of hydrophobic helices 5–7 (TH 5–7) is less well understood. Hydropathy analysis suggests that these helices might have the potential to insert as a second transmembrane hairpin, one side of which would be formed by a transmembrane helix 5 and the other side by a single transmembrane sequence formed by the combination of helices 6 and 7 (either of which alone is too short to span the bilayer) (2, 4). However, the sequence of the helix 5–7 region also has properties that make transmembrane insertion less likely. First, hydropathy suggests that helix 5 is not strongly hydrophobic (7). Second, helices 5 and 6 are separated by a fairly large loop region containing several polar and charged residues, which might result in a kinetic barrier to insertion of the hairpin. Last, helices 6 and 7 are separated by a kink formed by a Pro-Gly insertion.2

In previous studies, we found that the natural Trp in the center of helix 5 inserted into lipid bilayers (21, 22). In a recent paper, Senzel et al. (23) demonstrated that, at least in the state in which the T domain has an open pore, residues at the N-terminal of helix 5, along with the remainder of the N-terminal half of the T domain, can reach the trans side of the bilayer (i.e. the side opposite that from which the toxin has inserted) (23). They also found the C-terminal side of helix 5 remained on the cis side of the bilayer. This would imply that helix 5 spans the bilayer in their system. However, they did not define the boundaries of the membrane-inserted segments. Furthermore, the T domain is able to form multiple conformations, and so the topography they observed may correspond to only one of several conformations involved in the translocation process (see “Discussion”).

In this study, we performed a detailed topographical study of helices 5–7 using Cys-scanning mutagenesis followed by fluorescent labeling with Cys-reactive derivatives of bimane and BODIPY. Topography was then assessed by fluorescence and fluorescence quenching techniques previously developed in our laboratory (18–20). The results indicate that helices 5–7 undergo a conformational change in response to the conformational change that results in deep insertion of helices 8 and 9. As in the case of helices 8 and 9, helices 5–7 become more deeply inserted in the bilayer during this conformational change. In addition, the residues at the boundaries of the deeply inserted portions of helices 5–7 were identified. However, unlike helices 8 and 9, helices 5–7 do not appear to span the membrane in a stable fashion. Models for the various conformations formed by the membrane-inserted T domain and their potential impact on translocation are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Spin-labeled PCs (12S LPC, and TempOC), dioleoyl PC (DOPC), dimyristoyl PC (DMPC), 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 (23). Specific labeling on the Cys was confirmed by comparing the level of reactivity for 1 h. T domain mutant 282C did not label efficiently under these conditions. It was labeled under denaturing conditions, and then renatured as previously described (19). In all cases a high degree of specificity labeling on the Cys was confirmed by comparing the level of labeling attached to “wild type” (Cys-less) T domain.

**Fluorescence Measurements**—Fluorescence was measured at room temperature with a Spex 212 Fluorolog spectrophotometer operating in ratio mode. Unless otherwise noted, measurements were made in a semi-micro quartz cuvette (excitation path length 10 mm, emission path length 4 mm). The excitation and emission slit widths were used 2.5 and 5 mm, respectively. Trp fluorescence was measured with an excitation wavelength of 280 nm. Emission intensity was generally measured at wavelengths of 330 and 350 nm for 5 s, or emission spectra were measured (at a rate of 1 nm/s). Bimane fluorescence was measured with an excitation wavelength of 380 nm. Emission intensity was generally measured at wavelengths of 345 and 470 nm for 5 s, or emission spectra were measured. BODIPY fluorescence was measured for 10–15 s with an excitation wavelength of 488 nm and emission wavelength of 516 nm. In all cases, background intensities from samples lacking protein were subtracted from the intensities measured in the T domain-containing samples.

**Preparation of Model Membrane-incorporated T Domain—** Samples containing T domain were incorporated into sonicated small unilamellar vesicles (SUVs). Stock solutions of 70% DOPC, 30% DOPG or 70% DMPC, 30% DOPG (mol/mol) SUVs with a final concentration of 10 mM lipid were prepared as previously described (19). Typically, a 16-μl aliquot of SUVs was added to a cuvette containing 788–780 μl of 6.7 mM Tris-Cl, 167 mM acetate, 150 mM NaCl, (Tris acetate buffer), pH 4.5. To

2 G. Caputo and E. London, unpublished observations.
this. 0.46–1.8 µg (4–16 µl) of fluorescently labeled T domain, mixed with enough unlabeled protein to give a total of 1.8 µg of T domain, was added while mixing with a micro-stir bar. The final volume of each sample was 800 µl. For the bimane-labeled samples, fluorescence emission spectra were measured as described above. For the BODIPY-labeled samples, anti-BODIPY quenching was performed as described previously (18). To some samples, significant turbidity was observed after antibody addition. In such cases, T domain, lipid, and antibody concentrations were all decreased 2-fold. This largely eliminated turbidity.

Effect of Increasing T Domain Concentration and Addition of Serum Albumin on Bimane and BODIPY Assays—To examine the effects of T domain concentration and the addition of HSA on T domain behavior, bimane or BODIPY-labeled T domain was incorporated into 70% DOPC, 30% DOPG SUVs prepared as described above. Then, an aliquot giving a final concentration of either 11.4 µg/ml total T domain (usually 3–6 µl) or 5 µg/ml HSA (4 µl from a 1 mg/ml HSA solution) was added. After incubation for 5 min fluorescence intensity was measured as described above.

Relative Depth of Bimane-labeled Residues—For measurement of the depth of bimane-labeled residues within the bilayer, ether dilution SUVs that contained 30 mol % DOPG mixed with 70% PC composed of DOPC with or without 15 mol % active nitroxide-labeled lipid (12SIPC or TempoPC) were prepared (18). (The nitroxide content of nitroxide-labeled lipid was calibrated so that 15 mol % of lipids carried active nitroxide groups.) To prepare the SUVs, lipid mixtures dried from organic solvent were dissolved in 15 µl of 100% ethanol and then 671 µl of 6.7 mM Tris-Cl, 167 mM acetate, 150 mM NaCl, pH 4.5, was added rapidly while vortexing. Finally an aliquot (usually 14 µl) of bimane-labeled T domain mutant was added while stirring. The total volume of the sample was 700 µl with a final lipid concentration of 200 µM and final protein concentration of 2.3 µg/ml. Bimane fluorescence intensity was measured as described above. Subsequently, 4 µl from a 1 mg/ml solution of HSA was added to the sample to yield a final concentration of 5 µg/ml. The sample was mixed and allowed to incubate for 5 min after which time the fluorescence intensity was remeasured.

Effect of Removal of Hexahistidine (H6) Tag—The N-terminal H6 tag was removed from T domain mutants 311C, 293C, and 265C using thrombin as previously described (18). Removal of the tag was confirmed by SDS-polyacrylamide gel electrophoresis. These mutants were removed from T domain segments including residues of the helix 8 region (Fig. 3). The average 0.46–1.8 µg (4–16 µl) of fluorescently labeled T domain, mixed with enough unlabeled protein to give a total of 1.8 µg of T domain, was added while mixing with a micro-stir bar. The final volume of each sample was 800 µl. For the bimane-labeled samples, fluorescence emission spectra were measured as described above. For the BODIPY-labeled samples, anti-BODIPY quenching was performed as described previously (18). To some samples, significant turbidity was observed after antibody addition. In such cases, T domain, lipid, and antibody concentrations were all decreased 2-fold. This largely eliminated turbidity.

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Circular Dichroism Experiments—Circular dichroism (CD) measurements were made at room temperature in 1-mm path length quartz cuvettes using a Jasco J-715 CD instrument. Samples without lipid contained 5 µM wild type (WT) T domain in stock solution buffer diluted to 450 µl with 1 mM Tris-Cl, 15 mM NaCl, pH about 8. Samples with lipid contained 0.4 µM WT T domain dissolved in stock solution buffer diluted to 450 µl with 0.1 × Tris acetate, pH 4.5, buffer containing either 70% DOPC, 30% DOPG or 70% DMPC, 30% DOPG (mol/mol) sonicated SUV. Final lipid concentration was 800 µM. Reported spectra are the average of 125 individual wavelength scans. In control experiments, bimane Amax values in the P and TM states were not found to be greatly altered by the use of diluted buffer (data not shown). Intensities from background samples lacking peptide were subtracted from the protein spectra, and after conversion to molar ellipticity fractional α-helix was calculated using CONTIN, SELCON3, and CDSSTR (27).

Calculated helix content varied by roughly ±10% depending on protein choice, so the results from all three programs were averaged to obtain the final estimates of helix content.

RESULTS

Cys-scanning/Fluorescence Labeling Strategy—In previous studies, we have shown that the T domain in the shallowly inserted (P) and deeply inserted (TM) conformations can be distinguished by measuring the fluorescence of membrane-inserted T domains containing single-Cys residues labeled with bimane or BODIPY (18, 19). In almost all cases, neither introduction of the Cys residues, nor their labeling with these reagents seemed to perturb the low pH triggered conformational changes, membrane-insertion, or the balance between the P and TM conformations (18, 19). In this study, bimane and BODIPY labeling was extended to the hydrophobic helix 5–7 region of the T domain and adjacent sequences (Fig. 1). It should be noted that these helices are part of two uncharged, hydrophobic sequences. One extends from residues 272 to 289 and includes helix 5, and the other extends from about residues 300 to 317 and includes much of helix 6 and all of helix 7.

Emission Wavelengths of Bimane-labeled T Domain in the P Conformation—The fluorescence emission of T domain bimane-labeled on residues within helices 5–7 of in adjacent sequences was measured first. The λmax of bimane emission is red-shifted when exposed to an aqueous environment, and blue-shifted in a non-polar environment (18, 19, 28).

The λmax of bimane-labeled residues within the helix 5–7 region of membrane-inserted T domain is shown in Figs. 2–4. Previous studies have shown that the T domain spontaneously inserts into lipid bilayers under the low pH conditions used in this report (10, 18, 19, 21). Comparison of Figs. 2 and 3 shows there are significant differences between the bimane λmax values of membrane-inserted protein and those of the folded protein at neutral pH. Nevertheless, under conditions in which the membrane-inserted T domain forms the P conformation, bimane emission is relatively red-shifted throughout the helix 5–7 region (Fig. 3A). The average λmax of residues is about 467 nm, which is only about 2 nm more blue shifted than that of the residues of the helix 8–9 region when in the same conformation (18, 19). There is no clear distinction between λmax values for residues within the helices and those in the more hydrophilic sequences connecting helices. This pattern was also noted previously for helices 8 and 9 and their connecting loop in the P conformation (19). These results indicate that, like the helix 8–9 region, the helix 5–7 region lies in a relatively polar environment in the P conformation. However, there are a few residues (i.e. 293 and 317) that do show blue-shifted fluorescence.

For the residues mutated in this study, none of the Cys mutations perturbed the pH value at which the low pH-induced change in T domain conformation occurred (not shown). Of course, we cannot rule out local conformational perturbations due to mutation and/or labeling.
cence, and may be oriented toward the core of the lipid bilayer.

Emission Wavelengths of Bimane-labeled T Domain in the TM Conformation—There are distinct changes in bimane fluorescence when conditions are shifted to those in which the more deeply inserted TM conformation forms. Fig. 3B shows behavior in one such case, insertion into thinner bilayers (in which DMoPC replaces DOPC) (18). There is a blue shift in bimane fluorescence for almost all of the residues within helices 5, 6, and 7 relative to that in the P state. For residues within the hydrophobic segments in the helix 5–7 region the
The average blue shift is 5–6 nm. This is similar to the blue shift observed for residues in helices 8 and 9 when the protein converts from the P to TM state. Bimane-labeled residues within the kink connecting helices 6 and 7 (i.e. 307 and 309) also exhibit large blue shifts. In contrast, residues in the polar loop connecting helices 5 and 6 (5–6 loop) do not show a large blue shift. In fact, one residue within this loop, 293, shows a distinct red shift in the TM state. A lack of a large blue shift upon formation of the TM conformation was also previously observed for residues in the polar loop connecting helices 7 and 8 and in that connecting helices 8 and 9 (19).

The pattern of λ_max shifts in the TM state reveal the apparent boundaries of what appear to be two deeply inserted segments formed by the helix 5–7 region. These boundaries are defined by the edges of the clusters of residues that show a significant blue shift in bimane emission. In DMoPC-containing vesicles, the N-terminal boundary of the first segment, which overlaps helix 5, is not clearly demarcated, but it ends close to residue 290. The second segment extends from about residue 300 to residue 321. This segment not only spans both most of helix 6 and all of helix 7, it includes the kink between them. In other words, helices 6 and 7 can form a continuous hydrophobic segment that is potentially long enough, given the number of residues it contains, to span a lipid bilayer.

Bimane measurements were extended to membrane-inserted T domain under two other conditions previously shown to induce formation of the more deeply inserted (TM) conformation, namely when there are high T domain concentrations within the bilayer and when T domain interacts with a protein in a molten globule-like conformation (20). To achieve the latter condition, HSA was added to membrane-inserted T domain. (We previously demonstrated that HSA was the protein most effective in this regard (20).) As shown in Fig. 4, the overall λ_max pattern under both of these conditions is similar to that in DMoPC-containing bilayers. However, the boundaries of the membrane-inserted segments are slightly different than in DMoPC. Both at high T domain concentrations and in the presence of HSA the hydrophobic segment that overlaps helix 5 now seems to have an identifiable start, approximately near residues 270–272 and, as in DMoPC, extends to residue 290. At high protein concentrations, the second hydrophobic segment appears to be shorter, extending from about 305 (and thus not containing helix 6) to somewhere between 316 and 320. In the presence of HSA this segment extends roughly from 295 to 316 or 320.

**Exposure of BODIPY-labeled Residues to Anti-BODIPY in the P Conformation**—We further examined the topography of helices 5–7 by measuring the exposure of BODIPY-labeled mutants to externally added anti-BODIPY antibodies. Binding of anti-BODIPY to BODIPY groups results in quenching of BODIPY fluorescence. The intensity of fluorescence from BODIPY groups highly exposed to solution can be quenched 50–60% upon antibody binding (19). BODIPY groups that become buried within the bilayer show reduced quenching (19). In addition, because T domain does not form pores large enough for the antibody to pass through a bilayer (29), anti-BODIPY antibodies should also not quench BODIPY groups exposed on the internal (trans) surface of the bilayer. (The cis side is the
The quenching of BODIPY-labeled residues in the helix 5–7 region of membrane-inserted T domain is shown in Figs. 5 and 6. In the P conformation, almost all of the residues examined show roughly 50% quenching (Fig. 5A). This indicates that the entire helix 5–7 region resides close to the cis surface of the bilayer, consistent with the exposure of these residues to a polar environment suggested by bimane fluorescence. In addition, this behavior is similar to that previously found for BODIPY-labeled residues in helices 8 and 9 (19).

The only residues that show relatively weak quenching are 284, 288, and 293. These residues are at the C-terminal of helix 5 or in the loop region between helices 5 and 6. Interestingly, 293 is also the residue that exhibited the most blue shifted bimane fluorescence in the P conformation. Together, these results suggest part of the 5–6 loop and adjacent residues may be somewhat buried in the P conformation.

Exposure of BODIPY-labeled Residues to Anti-BODIPY in the TM Conformation—The BODIPY quenching pattern for T domain in the TM conformation is markedly different from that in the P conformation. First, the overall average quenching of residues within the helices drops by one-third to one-half, indicating a reduction in exposure to antibodies (Fig. 5B). This is seen most clearly in Fig. 5C, in which is presented the ratio of quenching in the P conformation to that in the TM conformation produced by insertion into DMoPC-containing vesicles. A ratio greater than one indicates a loss of antibody reactivity in the TM state. Fig. 5C also shows that a number of residues in the hydrophilic sequences flanking helices 5–7 do not become less reactive in the TM state. This indicates that these segments remain accessible to the external solution. As judged by BODIPY quenching, the boundaries of the membrane-inserted segment overlapping helix 5 appears to begin near 263 and end near 286, while the segment overlapping helices 6 and 7 begins near 302 and ends near residue 318. These values are in good, if not precise agreement with the boundaries determined by bimane fluorescence (see above). In addition, Fig. 5C shows that in the TM state residues 288 and 293 display a significant increase in reactivity with antibody in the TM state relative to that in the P state. This is in good agreement with the large red shift of λmax observed for bimane-labeled residue 293 upon conversion from the P to TM state. It should also be noted that the observation that these residues, which are in the loop between helices 5 and 6, remain exposed to antibodies on the cis side of the membrane is not consistent with the formation of a stable transmembrane hairpin in which helix 5 forms one transmembrane segment and a combination of helices 6 and 7 form another (see “Discussion”).

Comparison of Figs. 5C and 6 shows there are interesting similarities and differences between antibody quenching results for the TM state produced by insertion into DMoPC-containing vesicles, and the TM state formed at high T domain concentrations or by addition of HSA. The similarities include decreased exposure of residues within helices 5–7 to antibodies.
in the TM state, and increased antibody exposure of residues 288 and 293. One difference is that the boundaries of the segments that show decreased antibody accessibility in the TM state shift in the hydrophobic segment that overlaps helices 6 and 7. The shift in the boundaries are in agreement with the changes in the boundaries observed by bimane fluorescence under these conditions (see above). Additionally, the exposure of many residues within the hydrophobic segments to antibody is higher in the TM state formed at high T domain concentration or after the addition of HSA than it is in that formed upon insertion into DMoPC vesicles. This may mean that under these conditions the insertion in the TM state is not as deep, or as stable, as it is in DMoPC vesicles (see "Discussion").

Fluorescence Quenching of Bimane-labeled T Domain Mutants by Nitroxide-labeled Phospholipids—Both the bimane and BODIPY experiments strongly suggest that portions of helices 5–7 are more deeply inserted into the bilayer in the TM conformation than they are in the P conformation. To more directly confirm a change in depth within the bilayer is involved, we measured the depth of bimane-labeled residues using fluorescence quenching by nitroxide-labeled phospholipids (24, 25). To do this the degree of quenching of the fluorescence of a bimane-labeled residue by a lipid carrying a quencher group that locates deeply within the bilayer (12SLPC) was compared with the degree of quenching by a lipid with a shallowly located quencher (TempoPC). (The former molecule has a nitroxide group attached to the 12 carbon of one fatty acyl chain, while the latter molecule has a nitroxide attached to the polar headgroup.) If a labeled residue resides at a shallow depth, e.g. at the surface of the bilayer, then the shallow nitroxide will quench bimane fluorescence more heavily than the nitroxide located near the center of the bilayer. Conversely, if a residue inserts deeply in the bilayer, the bimane will be more heavily quenched by the deep nitroxide than by the shallow one. As a result, the ratio of fluorescence intensity in the presence of TempoPC to that in the presence of 12SLPC \( \left( \frac{F_{\text{tempoPC}}}{F_{\text{12SLPC}}} \right) \) is high when a fluorescent group is deeply buried in the bilayer.

Table I shows nitroxide quenching results obtained for a select number of bimane-labeled Cys mutants in the helix 5–7 region (those with Cys residues at positions 265, 284, 293, 311, and 320). In the P conformation, residue 293 is the most deeply buried of these residues, as shown by its having the highest \( \frac{F_{\text{tempoPC}}}{F_{\text{12SLPC}}} \) value. By the same criterion, residues 265 and 284 are located at the most shallow location, while residues 311 and 320 are at an intermediate depth (Table I). These results are consistent with the bimane \( \lambda_{\text{max}} \) results (Figs. 3A and 7).

To explore the depth of these residues in the TM conformation, HSA was added to each sample and fluorescence remeasured. This procedure eliminated variations of the amount of total fluorescence due to variations arising from the comparison of separately prepared samples. Upon addition of HSA, all of the residues except 293 become more deeply buried in the bilayer as judged by an increase in \( \frac{F_{\text{tempoPC}}}{F_{\text{12SLPC}}} \) (i.e. \( \Delta < 0 \)). This is in agreement with the observation that all of these residues undergo a blue shift under these conditions. In contrast, for residue 293 there is a decrease in \( \frac{F_{\text{tempoPC}}}{F_{\text{12SLPC}}} \).
Moreover, quenching of labeled residue 293 by both the shallow and deep nitroxides decreases. These changes are consistent with a movement of residue 293 away from the center of the bilayer and even away from contact with lipid. They are in agreement with the red shift of residue 293 upon addition of HSA. The overall correlation of $\lambda_{\text{max}}$ and nitroxide quenching data is summarized in Fig. 7.

Overall, the nitroxide quenching results indicate that upon conversion of the T domain from the P to TM conformation the changes in both bimane $\lambda_{\text{max}}$ and BODIPY quenching primarily reflect changes in the depth of the labeled residues within the bilayer. In addition, the mere observation of significant levels of quenching by both nitroxides, both before and after addition of HSA, confirm that T domain residues are in fact interacting with the lipid bilayer in both the P and TM states. (Nitroxides are short-range quenchers with an effective quenching radius on the order of 10–15 Å (24, 25).) Unfortunately, the nitroxide quenching data was relatively variable. This precluded the use of quenching to calculate precise depths by methods such as parallax analysis (24, 25).

**Secondary Structure of T Domain**—Circular dichroism (CD) spectra were obtained to determine whether the overall secondary structure of the T domain was affected by membrane insertion or T domain conformation. The CD spectrum of the protein in the far ultraviolet clearly shows that the secondary structure of the T domain is dominated by $\alpha$-helices (Fig. 8).

The fractional helix content of the T domain appears to increase significantly upon insertion into lipid, with little difference between the P and TM states (60–65% helix in solution at pH 8, and 80–90% helix for T domain membrane inserted in the P or TM states). Interestingly, an analogous increase in helix content upon membrane insertion was observed previously for the helical, membrane-inserting channel domain of colicin E1 (30).

**DISCUSSION**

**Helices 5–7 Can Exist in Two Distinct States Linked to the Conformations of Helices 8 and 9**—This study shows that helices 5–7 can exist both in shallowly and deeply inserted states. The shallowly inserted state is characterized by most residues in helices 5–7 being exposed to: 1) a polar environment and 2) external solvent, as shown by bimane fluorescence and accessibility to anti-BODIPY antibodies, respectively. In contrast, in the deeply inserted state bimane-labeled residues within the helices move into a more non-polar environment, and their reactivity with antibody when BODIPY-labeled is markedly reduced. Strikingly similar behavior was previously observed for helices 8 and 9 (19). Furthermore, the conditions that result in shallow or deep insertion of helices 5–7 are the same as those that yield shallow or deep insertion of helices 8 and 9. This means there is one state in which the entire C-terminal two-thirds of the T domain (helices 5–9) is close to the membrane surface (P state), and another in which it is deeply inserted (TM state). However, we do not know if the conditions leading to deep insertion independently act on helices 5–7 and 8–9, or if instead these segments undergo a tightly coupled conformational change. Another striking change linked to formation of the TM state is the increased exposure of at least some residues in the 5–6 loop to the external aqueous environment.

The structure of the deeply inserted T domain varies somewhat under different conditions. It appears from BODIPY quenching that the degree of burial of helices 5–7, and/or the fraction of T domain molecules in which helices 5–7 are stably buried within the core of the bilayer, is greater in thin bilayers than under the other conditions giving rise to deep insertion. However, bimane $\lambda_{\text{max}}$ suggests that the burial of hydrophobic

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**TABLE I**

| Bimane-labeled mutant/condition | $F_{\text{TempoPC}}/F_0^a$ | $F_{12SLPC}/F_0^b$ | $F_{\text{TempoPC}}/F_{12SLPC}^c$ | $\Delta^d$ |
|--------------------------------|----------------|----------------|---------------------------------|---------|
| 320C/DOPC                     | 0.52 ± 0.03     | 0.49 ± 0.05     | 1.07 ± 0.09                     | -0.04 ± 0.11 |
| 320C/DOPC + HSA$^e$           | 0.40 ± 0.03     | 0.36 ± 0.03     | 1.11 ± 0.13                     | -0.14 ± 0.10 |
| 311C/DOPC                     | 0.71 ± 0.08     | 0.65 ± 0.11     | 1.00 ± 0.13                     | -0.14 ± 0.10 |
| 311C/DOPC + HSA               | 0.69 ± 0.06     | 0.56 ± 0.08     | 1.24 ± 0.03                     | -0.14 ± 0.10 |
| 293C/DOPC                     | 0.56 ± 0.07     | 0.42 ± 0.03     | 1.24 ± 0.13                     | -0.14 ± 0.10 |
| 293C/DOPC + HSA               | 0.68 ± 0.12     | 0.57 ± 0.05     | 1.26 ± 0.23                     | 0.08 ± 0.25  |
| 284C/DOPC                     | 0.66 ± 0.07     | 0.77 ± 0.08     | 0.88 ± 0.07                     | -0.16 ± 0.09 |
| 284C/DOPC + HSA               | 0.69 ± 0.05     | 0.68 ± 0.04     | 1.04 ± 0.06                     | -0.05 ± 0.03 |
| 285C/DOPC                     | 0.56 ± 0.09     | 0.58 ± 0.08     | 0.99 ± 0.09                     | -0.05 ± 0.03 |
| 285C/DOPC + HSA               | 0.64 ± 0.05     | 0.62 ± 0.08     | 1.04 ± 0.05                     | -0.05 ± 0.03 |

$^a$ Fraction of unquenched fluorescence in samples containing either a shallow quencher ($F_{\text{TempoPC}}$) or a deep quencher ($F_{12SLPC}$) as compared to a sample containing no quencher ($F_0$). Values shown are the averages of 6–10 samples and standard deviations.

$^b$ Equal to $(F_{\text{TempoPC}}/F_0)/F_{12SLPC}/F_0$ and related to the depth of the residues within the bilayer (Ref. 18, see text). Ratios were calculated for sets of samples containing the different quenchers that were prepared at the same time. The average and standard deviation of those ratios is shown.

$^c$ Delta is the change in $(F_{\text{TempoPC}}/F_0)/F_{12SLPC}/F_0$ following the addition of HSA. Standard deviation shown is for the variability of $\Delta$ for individual samples before and after HSA addition.

$^d$ After measuring fluorescence, 5 μg/ml HSA was added and fluorescence was remeasured.

**FIG. 7.** Correlation between bimane emission $\lambda_{\text{max}}$ and depth parameter ($F_{\text{TempoPC}}/F_{12SLPC}$) obtained from nitroxide-lipid quenching experiments. Triangles, T domain inserted in P conformation; circles, T domain inserted in TM conformation by addition of HSA. Data shown is derived from Figs. 3 and 4 and Table I.
residues is similar in all the different conditions giving the TM state. One explanation of this discrepancy is that transient fluctuations allow relatively deeply buried BODIPY-labeled residues to become sufficiently exposed to solution to bind antibody when the deeply buried state forms at high T domain concentrations or in the presence of HSA. Another explanation is that there are small differences in the structure of bimane and BODIPY-labeled T domain molecules arising from the effects of labeling.

Boundaries of Membrane-inserted Segments in the Helix 5–7 Region in the Deeply Inserted State—These studies also identified the approximate boundaries of the membrane-inserted segments of helices 5–7 in the deeply inserted (TM) state. Although the exact residues at the boundaries vary a bit depending on the conditions in which the TM state forms, in general they are close to the boundaries of the hydrophobic sequences overlapping helices 5–7. For the deeply inserted state forming in DMoPC-containing vesicles, bimane fluorescence indicates one deeply buried segment extending from residues 271 to 290, largely overlapping helix 5, and a second extending from residues 300 to 320, which includes much of helix 6, helix 7, and the intervening kink (residues 305–309). Similar values are obtained from analysis of BODIPY fluorescence.

The N-terminal boundary of the deeply inserted segment that includes helix 5 is the most poorly defined by the data. For T domain inserted into DMoPC-containing vesicles, the region extending from residues 263 to 270 appears buried in the TM state. However, for the TM state forming at high protein concentrations or in the presence of HSA, it is possible that the boundaries of the T domain sequence from 265 to 272 is itself a bit ambiguous, as it is composed of uncharged but mainly polar residues.

In evaluating the relationship between the helices and hydrophobic segments the increase in helix content upon membrane insertion must be considered. The boundaries of helices 5–7 in the membrane-inserted state are likely to be different than those determined from the crystal structure of the native protein in solution.

Interpretation of BODIPY Quenching—A number of factors complicate analysis of BODIPY quenching. A difference in % quenching for two residues having different degrees of exposure to solution could potentially result from differences in how tightly antibody binds to BODIPY or differences in how rapidly antibody binds. However, in every case tested, it was found that the amount of antibody used was an excess that gave maximal quenching, and that quenching was rapid, being nearly complete in just a few minutes (not shown). The amount of quenching upon antibody binding could also potentially be affected by differing spectroscopic properties of BODIPY groups at different labeling sites. In particular, if different residues have different quantum yields prior to antibody binding (for example, because they are located in environments with different polarities), but all have the same quantum yield when bound to antibody, then the % quenching would be a function of the initial fluorescence rather than just exposure to antibody. However, BODIPY fluorescence is relatively insensitive to polarity, greatly reducing the possibility of such artifacts (31, 32). Indeed, prior to antibody addition, the difference between intensity of BODIPY fluorescence in the P and TM states was only 10–20% for most residues (not shown). This was too small to explain the decrease in quenching in the TM state as being due to an artifact, and “correcting” for any changes in initial fluorescence had little impact on the results (not shown). In any case, the most convincing evidence that the BODIPY quenching primarily reflects exposure to solution is its close agreement with the results obtained from bimane $\lambda_{\text{max}}$.

For these reasons, the most probable interpretation of decreased quenching in the deeply inserted state is that there is a reduction in the number of exposed BODIPY groups, which results in a decrease in the fraction of BODIPY-labeled molecules that are antibody bound. We attribute the residual quenching of residues that appear deeply buried in the TM state to be due to a population of T domain molecules that fail to insert deeply and/or stably. We previously found that about half of the T domain molecules underwent the transition to the TM state (19).

Model for the Conformation of Helices 5–7 in the Shallowly Inserted (P) State—Combining the results of bimane and BODIPY fluorescence allows formulation of a model for the conformation of the helix 5–7 region within a lipid bilayer (Fig. 9). In the shallowly inserted P form it appears that the helix 5–7 region lies at the cis surface of the membrane. This is the only model consistent with the exposure of residues throughout this region both to a polar environment and to externally added antibodies. Since similar behavior was found for helices 8 and 9 in this state (19), we propose that almost the entire helix 5–9 region of the T domain is shallowly inserted in the P state (model a).

The Conformation of Helices 5–7 in the Deeply Inserted TM State: Helices 5–7 Do Not Form a Stable Transmembrane Hairpin but May Be Transmembraneous Under Some Conditions—

4 Because the level of labeling by BODIPY was somewhat variable, to insure that the antibody was in excess samples with similar amounts of BODIPY fluorescence were used. This should represent about equal amounts of label, because BODIPY quantum yield is insensitive to environment.

5 It should be noted that unlike our previous study on helices 8 and 9, bimane $\lambda_{\text{max}}$ values in the TM state for residues in the helix 5–7 region have not been calculated after adjustment for incomplete insertion. The reason is that, unlike residues in helices 8 and 9, which exhibit a consistent 50% decrease in BODIPY quenching in the TM state relative to the P state, the fractional change in BODIPY quenching upon the P to TM conformational change, and thus perhaps the amount of deep insertion, is more variable for residues in the helix 5–7 region.
As noted above, helix 5 and the helix 6/7 combination form two hydrophobic segments that could theoretically insert as a transmembrane hairpin connected by a solvent exposed 5–6 loop (model c). However, the BODIPY quenching data shows that a stable transmembrane hairpin is not present in the deeply inserted state. If it was, the 5–6 loop residues would have become inaccessible to externally added antibodies. Instead, the behavior of helices 5–7 and the 5–6 loop are consistent with several alternate topographical models. This is true because antibody quenching of BODIPY-labeled residues does not distinguish between structures in which exposed residues locate stably on the cis side of the bilayer and those in which they move back and forth between the cis and trans sides due to a dynamic equilibrium. Solution-exposed residues need only locate on the cis surface for a short period of time for antibody binding to trap them on the cis side of the membrane and result in strong quenching.

With this in mind, Fig. 9 illustrates possible conformations of helices 5–7 in the more deeply inserted state. To be consistent with previous data a transmembrane orientation is shown for helices 8 and 9, with the loop between helices 7 and 8 exposed on the cis side of the bilayer (19). One possible conformation is one in which helices 5–7 insert more deeply than in the P state, but remain close to the cis side of the membrane rather than form transmembrane structures (model b). This is the only model consistent with the data if a single, stable conformation is present in the TM state. The non-transmembrane orientation of these helices could reflect unfavorable energetics or a kinetic barrier that prevents translocation of the polar and charged residues in the 5–6 loop across the membrane.7

The alternative is that there is a dynamic equilibrium between transmembrane and non-transmembrane conformations. If this is the case, there are three topographical arrangements that might exist in equilibrium with model b and/or each other. At one extreme is the possibility that an unstable transmembrane helical hairpin forms (model c). Models in which only helices 6/7 (model d) or only helix 5 (model e) form unstable transmembrane segments are also consistent with strong BODIPY quenching of helix-flanking hydrophilic segments. Notice that to be consistent with the bimane data, even the non-transmembrane hydrophobic segments in models d and e would have to be deeply inserted relative to their insertion in the P state. In model d the non-transmembrane helix 6/7 segment would be closer to the cis side of the bilayer. In model e, a non-transmembrane helix 5 would be closer to the trans side of the bilayer.

**Additional Information on Helix 5–7 Topography from Previous Studies and the Potential Role of Conformational Flexibility in Translocation**—Previous studies have not fully defined the topography of helices 5–7. Based on the DT crystal structure and hydrophy analysis, it was originally predicted that helices 5–7 would insert as a helical hairpin, together with a hairpin formed by helices 8 and 9 (2, 4). Photolabeling studies did not detect extensive interaction of this part of the protein with lipid (33). On the other hand, consistent with deep insertion of this entire region, Cabiaux et al. (17) identified a protected fragment spanning residues 265–375 (i.e., essentially all of helices 5–9) when DT inserted into asolectin vesicles at low pH was treated with a nonspecific protease.

Recent studies by Senzel et al. (23) on T domain topography in planar lipid bilayers present convincing evidence that in addition to helices 8 and 9, helix 5 spans the bilayer. In their model, helices 6 and 7 lie close to the cis surface while helices 1–4 are on the trans side of the membrane (23, 34). This is equivalent to model d, and is consistent with our data if it exists in equilibrium with other conformations under our conditions. However, the planar bilayer studies only detect T domain molecules that are in the open channel state. If the number of molecules in the open channel state is only a fraction of the total T domain molecules this conformation may not be representative of that of the bulk of T domain molecules. Our experiments tend to probe the average behavior of T domain molecules.

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6 We still use the term TM state for the deeply inserting conformation, but this name should be understood to only refer to the transmembrane orientation of helices 8 and 9.

7 It should be noted that we cannot rule out the possibility that the BODIPY labels somehow affect the ability of the helix 5–7 region to take on a transmembrane orientation. However, it seems unlikely that BODIPY could strongly inhibit transmembrane insertion, as it is a relatively hydrophobic group (1).
The possibility that the helix 5–7 region can adopt more than one conformation in the deeply inserted state is intriguing, and could play an important role in translocation of the A chain. For example, the energy gained by conversion of the T domain in some metastable membrane-inserted state to a more stable one could help pull the A chain through the bilayer. One possibility is that after an initial deep insertion as shown in models b or c, in which the A chain would be on the cis side of the bilayer, there is a shift to the conformation shown in model d, in which it is on the trans side. Starting from model c, this would require a seemingly improbable 180° flip of helix 5. However, it should be noted there is a precedent for such a flip in the action of Sec G, a component of the bacterial translocon, upon its interaction with Sec A (35). Examining the possibility that the T domain has multiple, interconverting deeply inserted states with distinct roles in translocation will be a goal of our future studies.

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