Membrane Topography of the T Domain of Diphtheria Toxin Probed with Single Tryptophan Mutants†

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ABSTRACT: The membrane insertion and translocation of diphtheria toxin, which is induced in vivo by low pH, is thought to be directed by the hydrophobic α-helices of its transmembrane (T) domain. In this study the structure of membrane-associated T domain was examined. Site-directed mutants of the T domain with single Trp residues were prepared at the two naturally occurring positions, 206 (near the N-terminal end of helix TH1) and 281 (within helix TH5), as well as at three residues in helix TH9, in which the substitutions F355W (near the N-terminal end of TH9), I364W (close to the center of TH9), and Y375W (near the C-terminal end of TH9) were made. All these mutants were found to undergo the low-pH-induced conformational change observed with wild-type T domain and insert into model membranes at low pH. The location of Trp residues relative to the lipid bilayer was characterized in model membrane vesicles by fluorescence emission and by quenching with nitroxide-labeled phospholipids. In TH9, residue 375 was shallowly inserted, residue 364 deeply inserted, and residue 355 located at an intermediate depth. Residues 206 and 281 exhibited moderately deep insertion. It was also found, in agreement with our previous study using bimane-labeled protein (Wang et al. (1997) *J. Biol. Chem. 272*, 25091−25098), that TH9 switches from a relatively shallowly inserted state to a more deeply inserted state when the concentration of the T domain in the membrane is increased or the thickness of the membrane bilayer is decreased. In particular, the depth of residue 355 was found to increase under the conditions giving deeper insertion. In contrast, residue 375 remained shallowly located in both states, as predicted from its location on the polar C-terminus of TH9. It is concluded that TH1 and TH5 insert into the lipid bilayer in both T domain conformations. In addition, Trp depths suggest that even in the shallowly inserted state there is a significant degree of insertion of TH9. These results suggest regions of the T domain in addition to the hydrophobic TH8/TH9 hairpin insert into membranes. Models for the structure of the membrane-inserted T domain are discussed.

The process of protein translocation across a lipid bilayer is poorly understood at the molecular level. We are interested in the regulation of this process, using diphtheria toxin as a model protein. Diphtheria toxin, a cytotoxic protein of 535 amino acid residues, is secreted by *Corynebacterium diphtheriae* (*1*). The crystal structure shows that the toxin is composed of three distinct structural domains, designated the receptor binding (R, residues 382−535), catalytic (C, residues 1−193), and transmembrane (T, residues 194−381) domains (*2*−*4*). In vivo, each domain has a discrete function, and they work in concert to enter and kill cells. The R domain hijacks an EGF-like precursor protein (*5*), and the crystal structure of a fragment of this receptor complexed with the toxin was recently solved (*6*). This association allows diphtheria toxin to undergo receptor-mediated endocytosis and reach the lumen of endosomes. The acidic pH of the endosomal lumen causes a partial unfolding of the toxin, exposing hydrophobic regions on the protein and thus triggering membrane insertion (*7*). The T domain mediates the membrane insertion of the toxin and is thought to assist in maneuvering the C domain through the membrane. Cell death is dependent on translocation of the C domain into the cytosol, where it catalyzes the ADP ribosylation of elongation factor 2, consequently inhibiting protein synthesis (*1*).

The T domain is primarily comprised of 10 α-helices, (named TH1−9 and TH5′). Two helices that are particularly long and hydrophobic, TH8 and TH9, are part of a hairpin joined by a loop (TL5) containing acidic residues (*2*−*4*). It has been proposed that this region of the T domain guides its insertion into the endosomal membrane and that TH8 and TH9 take on a transmembraneous conformation after protonation of the residues in the TL5 loop (*2*, *8*, *9*).

For this reason recent studies on the T domain have addressed the question of the topology of the membrane-inserted helices, in particular for TH8 and TH9. The minimal

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channel-forming domain of the T domain has been determined and shown to be a 61 residue stretch encompassing TH8−9 (*10*). This and other channel-forming studies on the T domain are consistent with a model in which the TL5 loop that connects TH8 to TH9 passes through the bilayer to the trans side (*9*, *11*−*13*). Proteolysis studies found TH8−9 to be the most protected region of the T domain (*14*−*16*). Electron spin resonance studies have determined that TH9 retains a helical structure upon membrane binding. These studies also determined which sides of this helix are aqueous or lipid facing. On the basis of the ESR data, a transmembrane structure was proposed for helices TH8 and TH9 (*17*, *18*).

Despite these studies, the behavior of the membrane-inserted TH8/TH9 hairpin and the remainder of the T domain is not well-understood. For example, recent studies of pore properties have been interpreted in terms of a flexible, nonhelical structure for the TH8/TH9 hairpin (*19*). In addition, we recently found that the membrane-bound TH8/TH9 hairpin can exist in two conformations, one of which is much more deeply inserted than the other (*20*). It was shown that the balance between these two conformations is regulated by the protein concentration in the bilayer and by bilayer width, and it is unclear whether some non-transmembrane conformation T domain was present in previous studies. Even less is known about the topography of the remainder of the T domain (see Discussion).

We have previously examined the overall membrane insertion of whole diphtheria toxin using Trp restricted to either the A chain (C domain) or the B chain (T plus R domains) (*21*). This report analyzes the membrane-inserted positions of five individual Trp residues located in helices TH1, TH5, and TH9, using fluorescence emission properties and fluorescence quenching. The regulation of T domain insertion into model membranes by protein concentration in the bilayer and by the thickness of the bilayer was also examined. The results confirm that the T domain inserts in at least two different conformations and indicate that TH1 and TH5 insert into the membrane bilayer in both of these conformations.

EXPERIMENTAL PROCEDURES

Materials. Spin-labeled PCs (SLPC),[1] dimyristoleoyl PC (DMoPC), dioleoyl PC (DOPC), and dioleoyl PG (DOPG) were purchased from Avanti Polar Lipids (Alabaster, AL). The purity of the phospholipids was checked by TLC on silica gel plates, as described previously (*22*). No impurities were detected in the lipids after the plates were sprayed with 40% sulfuric acid (v/v) and charred. The concentrations of the spin-labeled PCs, DMoPC, DOPG, and DOPC were determined by a phosphate assay after total digestion or by dry weight (*22*). For DMoPC the manufacturer's specifications were used. The actual nitroxide content of the nitroxide-labeled lipids was calculated from the intensities of the doubly integrated ESR spectra, as described previously. Alternatively, the concentration of the spin-labeled lipids was assayed by determining the percent of nitroxide-labeled lipid that had to be incorporated into multilamellar vesicles to give the same quenching of anthroyloxy fatty acids as that found previously for multilamellar vesicles containing 15% nitroxide-labeled lipids (*22*). The ratio of nitroxide groups to lipid was generally found to be in the range 0.6−0.9. All other chemicals were reagent grade.

T Domain Protein Expression and Purification. Wild-type T domain contains two endogenous Trps, at positions 206 and 281. Single mutations in wild-type T domain, W206F and W281F, were constructed to create two mutants with single Trp. To create additional single-Trp mutants, both native Trps were mutated to Phe and then an additional Trp was introduced into helix TH9. These mutants are W206F,-W281F,F355W; W206F,W281F,I364W; and W206F,W281F,-Y375W. For simplicity all mutants will be referred to only by the position of the Trp. The methods used to produce the plasmids for the expression of wild-type T domain protein and single-Trp mutants are detailed in Zhan et al. (*17*). The proteins expressed contain an NH_2-terminal hexahistidine (His_6) tag fused to T domain residues 202−378. When the His_6 tag is removed, four additional residues are left at the NH_2 terminus: Gly-Ser-His-Met. When the His_6 tag was not removed, 21 additional residues were left at the NH_2 terminus: Met-Gly-Ser-(His_6)-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met. (The whole diphtheria numbering system is used in this report, so these additional residues are not counted in the numbering scheme (*17*).)

The purification procedure was basically that described in the pET system manual from Novagen, Inc. (Madison, WI). Briefly, the protein was cytoplasmically expressed in *Escherichia coli* (*E. coli*) cultures, and the cells were harvested and then disrupted by sonication. Cell debris was removed by centrifugation, and the supernatants were collected and loaded on 2−3 mL nickel chelate affinity columns (Novagen). A stepwise gradient of imidazole was used to wash and then elute the protein, as described in the pET system manual. The eluants were dialyzed against 20 mM Tris-HCl buffer, pH 8.0, for 16 h at 4 °C with two changes of buffer. The protein samples were then concentrated with a Centriprep 10 (molecular weight cutoff, 10 000; Amicon).

Purity was >90−95% as judged by SDS gel electrophoresis. The final protein concentration was determined using the Coomasie Plus Protein Assay Reagent from Pierce (Rockford, IL), using bovine serum albumin as the standard, and from absorbance at 280 nm using $\epsilon = 18\ 200\ M^{-1}\ cm^{-1}$, and was converted to micrograms per microliter using an approximate molecular weight of 20 000. Purified T domain was stored at 4 °C in 20 mM Tris, pH 8.0.

Fluorescence Measurements. Fluorescence was measured with a Spex 212 Fluorolog spectrofluorometer operating in ratio mode at room temperature. Measurements were made in a semi-microcuvette (excitation path length, 10 mm; emission path length, 4 mm). The excitation and emission slits were 2.5 and 5 mm (band-passes of 4.5 and 9 nm, respectively). Lipid binding experiments were performed with an excitation wavelength of 280 nm, and the emission was measured at 330 and 350 nm. Fluorescence quenching experiments were performed with an excitation wavelength of 295 nm. Controls showed no significant difference in

[1] Abbreviations: DMoPC, di $C_{14:1\Delta9c}$-phosphatidylcholine; DOPC, di-$C_{18:1\Delta9c}$-phosphatidylcholine; DOPG, di-$C_{18:1\Delta9c}$-phosphatidylglycerol; PC, phosphatidylcholine; SDS, sodium dodecyl sulfate; 5 or 12 SLPC, 1-palmitoyl-2-(5 or 12- doxyl)stearoylphosphatidylcholine; SUV, small unilamellar vesicle; TempoPC, 1,2-dioleoyl-*sn*-glycero-3-(4-(*N*,*N*-dimethyl-*N*-(2-hydroxyethyl)ammonium)-2,2,6,6-tetramethylpiperidine-1-oxyl).

quenching when the excitation wavelength was 280 nm. Trp emission spectra were measured from 320 to 360 nm. Emission intensity was measured for 1 s, and emission spectra were measured in 1 nm intervals. Emission intensity for the fluorescence quenching experiments was measured at the emission wavelength maximum of the sample without any nitroxide quencher, which varied for each mutant. The background intensities and/or spectra from samples without T domain were subtracted from those with T domain in all cases. Unless otherwise noted, all measurements were made at room temperature.

Effect of pH on T Domain Fluorescence. Aliquots (2−40 μL) containing 4 μg of T domain were diluted to 600 μL with PBS (10 mM sodium phosphate, 150 mM NaCl), pH 8, to give a final protein concentration of 0.33 μM. These samples were gradually titrated with 1−6 μL aliquots of 0.8 M acetic acid to pH 4.5 and then with aliquots of 0.9 M HCl to pH 3.5. After addition of each aliquot, the samples were mixed by gentle inversion and incubated for 1 min, pH was measured with a microelectrode (Microelectrodes, Inc., Londonderry, NH), and then Trp fluorescence intensity was measured at excitation emission of 280 nm and emission wavelengths of 330 and 350 nm.

Preparation of SUV. Sonicated SUVs were used for all experiments in which model membranes were present with the exception of the depth measurements (see below). To prepare sonicated SUVs the appropriate concentrations of stock solutions of the desired lipids (in ethanol or $CHCl_3$) were added to a glass tube. The lipids were vortexed and then dried under a stream of N_2, followed by 1 h of drying in vacuo. The lipids were then reconstituted to a concentration of 10 mM with PBS, pH 7−7.5, and sonicated in a water bath sonicator (Laboratory Supplies Co., Hicksville, NY; model no. G112SP1T), until optically clear. The lipid composition of the SUV was 30% DOPG and 70% of either DOPC or DMoPC (mole/mole).

Lipid Binding to T Domain. Lipid binding experiments started with a pH 4.0 sample prepared with 4 μg of T domain diluted to 600 μL with 50 mM acetate, 150 mM NaCl, pH 4.0, and placed in the semi-micro-fluorescence cuvette. After measurement of fluorescence, small aliquots (1−7 μL) of 10 mM sonicated SUVs or SUVs diluted with PBS to 1 mM were titrated into the samples, to obtain various lipid concentrations up to 500 μM.[2] After each addition, the sample was stirred with a microstirbar and then incubated between 30 s and 1 min before remeasuring fluorescence. Fluorescence readings were taken with an excitation wavelength of 280 nm and emission wavelengths of 330 and 350 nm. The dilution by lipid was taken into account when the final lipid concentration of the samples was calculated. The effect on the pH of the PBS added with the lipid (no more than 35 μL by the end of the experiment) was negligible.

Preparation of Samples for Measurement of Trp Depth. Samples for fluorescence quenching were prepared using ethanol dilution SUV (*21*). (It should be noted that the conformational behavior of the T domain bound to sonicated and ethanol dilution vesicles is very similar (*20*).) In general, vesicles were prepared by adding the desired amount of lipid from stock solutions in ethanol or $CHCl_3$ to a glass tube and then by adding 60 μL of $CHCl_3$ and vortexing well. The lipids were then dried first under N_2 and then in vacuo for 1 h. To reconstitute the dried lipids, 10 μL of 100% ethanol was added to each sample tube and vigorously vortexed to redissolve the lipid. Next, the appropriate volume of 50 mM acetate, 150 mM NaCl buffer, pH 4.0, generally 525−599 μL, depending on the volume of protein used, was rapidly added, creating the SUV. A (1−75 μL) aliquot containing 4 μg of T domain was then added (final protein concentration of 0.33 μM). In the low protein-to-lipid ratio conditions (protein-to-lipid molar ratio 1:1000) the final lipid concentration was 333 μM. For the high protein-to-lipid ratio conditions (protein-to-lipid molar ratio 1:150) the final lipid concentration was 50 μM. In all cases the final pH was 4 and the final volume was 610 μL. For the samples lacking quencher (F_0) the lipid mixture used was 30% DOPG and 70% DOPC or DMoPC (mole/mole) as appropriate. Samples containing the spin-label quencher were comprised of 30% DOPG, 55% PC (DMoPC or DOPC, plus a small amount of inactive spin-label PC), and 15% active SLPC (Tempo PC, 5SLPC or 12SLPC) (mol/mol) (*20*).[3] The fraction of fluorescence unquenched (F_{TC}/F_0; F_5/F_0; or F_{12}/F_0) was calculated from the ratio of fluorescence intensity in the presence of quencher (F_{TC}, F_5, or F_{12}) for the TempoPC, 5SLPC, and 12SLPC quenchers, respectively, to that in its absence (F_0). Experiments to measure depth were performed 3−8 times, usually with duplicate samples. The minimum number of samples was six for all depth experiments.

Effect of Removal of the His_6 Tag. Control experiments were performed on the wild-type and some mutant T domain proteins to determine if the presence of the His_6 tag had any effect on the protein conformation. The His_6 tag was removed by thrombin proteolysis as described in the Novagen, Inc., manual and removal was confirmed by SDS gel electrophoresis. No difference was observed upon removal of the His_6 tag in lipid binding experiments. In addition, the presence or absence of the His_6 tag had no effect on the pH dependence of fluorescence or the Trp depth determined from fluorescence quenching experiments, even in the case of the W206 mutant, which carries a Trp in TH1 adjacent to the His_6 tag. Furthermore, no effect of the His_6 tag was observed in previous fluorescence studies of labeled Cys mutants (*20*). Therefore, unless otherwise noted, the His_6 tag was not removed.

RESULTS

Fluorescence Properties of Wild-Type and Mutant T Domain Proteins: Effect of pH on T Domain in Solution. Trp fluorescence was used to probe the structure of the membrane-inserted T domain. The behavior of mutants with a single Trp at residues at 206, 281, 355, 364 and 375 was tested. The T domain mutants were first examined to see if they would undergo the low-pH-induced conformational change observed for both whole toxin (*23*) and isolated T

[2] It should be noted that the T domain may cause vesicle fusion at high concentrations, as suggested by an increase in turbidity, and thus the final vesicles structure may be closer to that of large unilamellar vesicles.

[3] Due to the presence of nitroxide-labeled lipid, there was a small difference in the average acyl chain length in the DMoPC-containing samples which depended on the amount of inactive nitroxide. This difference should be too small to affect T domain conformation (*20*).

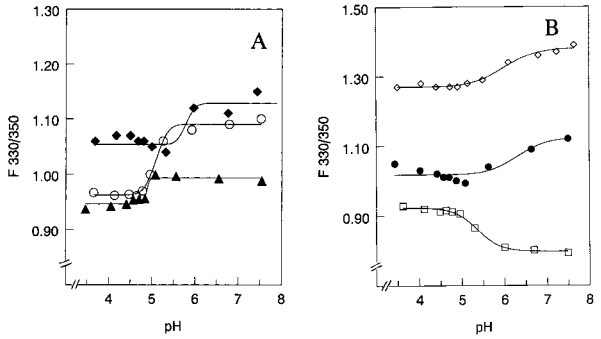

FIGURE 1: Effect of pH on T domain fluorescence in solution. F 330/350 is the ratio of emission intensity at 330 to that at 350 nm. (A) Fluorescence of wild-type T domain (○) and single-Trp mutants in TH1, W206 (▲) and in TH5, W281 (♦). (B) Single-Trp mutants in TH9, W355 (●), W364 (◇), and W375 (□). T domain concentration was 0.33 μM.

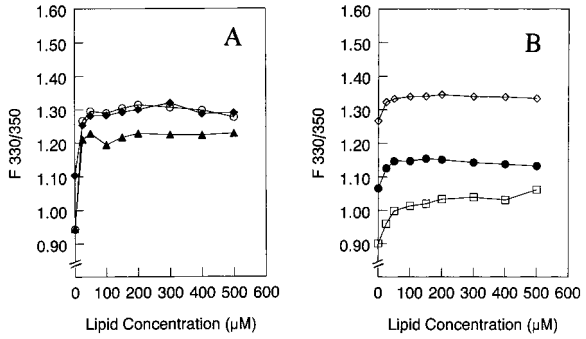

FIGURE 2: Binding of T domain to DMoPC-containing model membranes at low pH. Samples with 0.33 μM T domain at pH 4 were titrated with small aliquots of 30%/70% DOPG/DMoPC (mole/mole) SUV. (A) Fluorescence of wild-type T domain (○), W206 (▲), and W281 (♦). (B) Fluorescence of W355 (●), W364 (◇), and W375 (□).

domain (*9*). This pH-dependent conformational change is the crucial step for inducing membrane penetration in cells (*7, 24*). The conformational change was monitored by measuring Trp fluorescence emission wavelength shifts. These shifts were assayed by the ratio of Trp fluorescence emission intensity at 330 nm relative to that at 350 nm (*25*).

Figure 1 shows the effect of pH on the 330/350 emission ratio for T domain in solution. Interestingly, the differences in Trp emission for the different mutants at pH 7 is close to that which would be predicted from the crystal structure of the toxin (*2*−*4*). The Trp introduced at residue 375, which would be the most exposed to solvent of all the Trp we studied according to the crystal structure, showed the most red-shifted fluorescence (i.e., the lowest 330/350 emission ratio) of all the Trp residues studied, consistent with a location in a polar environment. The Trp introduced at residue 364, which is the most deeply buried according to the crystal structure, showed the most blue-shifted fluorescence (highest 330/350) of all the Trp studied, consistent with a location in a nonpolar environment.

For the wild-type T domain, a change in the 330/350 ratio indicative of a conformational change was observed at low pH. The midpoint of the conformational transition was about pH 5.2 (Figure 1A), close to the value previously detected by measuring the binding of the 2-*p*-toluidinylnaphthalenesulfonic acid (*9*). The decrease in the 330/350 ratio at low pH can be an indicator of exposure of Trp to a more polar environment, which is consistent with increased exposure of buried sites to the aqueous solution at low pH observed with both whole toxin and isolated T domain (*9, 23, 26*).

The mutant T domain proteins also exhibited fluorescence changes indicative of the low-pH conformational transition. The 330/350 ratio decreases at low pH for the W206, W281, W355, and W364 mutations, consistent with these residues becoming more exposed to solution at low pH. In contrast, for W375, which is unusually solvent exposed at pH 7 (see above), the 330/350 ratio increases at low pH, consistent with a decrease in solution exposure. All Trp mutants show the fluorescence change in the pH range 5−6 (Figure 1), suggesting the low-pH-induced conformational change occurs under conditions very similar to that of wild-type protein. Small differences in the transition pH for different mutants imply that either the conformational change occurs at slightly different pH values within different segments of the protein or that there are small differences in the pH stability of the native and low-pH conformation in the various mutants.

Binding of T Domain to DMoPC-Containing Model Membranes at Low pH. The interaction of the T domain mutants with model membrane vesicles at low pH was examined both to see if they bound to vesicles with the same affinity as wild-type protein and to evaluate the effect of lipid vesicles on the environment of the Trp residues. Lipid binding was monitored by dependence of the Trp fluorescence emission 330/350 ratio on increasing lipid concentration. Figure 2 shows lipid binding curves for the wild-type and the single-Trp mutants of the T domain binding to DMoPC-containing vesicles, which form relatively thin bilayers, and in which our previous studies have indicated that deep insertion of the TH8/TH9 helical hairpin is obtained (*20*). T domain binding to lipid vesicles was found to be rapid, basically complete by 20 s (data not shown). For all five Trp mutants there was a blue shift in fluorescence upon lipid binding, consistent with transfer of these residues to a less polar environment upon binding to lipid. (However, it should be noted the fluorescence behavior of the W364 mutant was somewhat variable in both these and the DOPC-containing samples.) All of the binding curves were similar, showing tight binding that was half-maximal at no more than 25 μM lipid. This shows the wild-type and all mutants have similar lipid binding properties at low pH.

Binding of T Domain to DOPC-Containing Model Membranes at Low pH. Somewhat more complex binding curves were detected in DOPC-containing model membranes (Figure 3). For the W206 and W375 mutants the Trp fluorescence blue-shifted when the T domain associated with the DOPC-containing vesicles. However, residue 364 underwent a small red shift upon addition of lipid. Even more striking, residue W355, and to a lesser degree W281, exhibited at first an increase (blue shift) in the 330/350 ratio (reaching a maximum value close to the value observed in DMoPC vesicles) and then a decrease (red shift) as lipid concentration increased.

This behavior can be explained by our previous observation that in DOPC-containing model membranes the conformation of the T domain depends on its concentration in the bilayer. At a high protein-to-lipid ratio there was deeper membrane insertion of the TH8/TH9 region than at low protein-to-lipid ratio as judged by the location of a bimane probe attached to residue 356 (*20*). The lipid titration curve

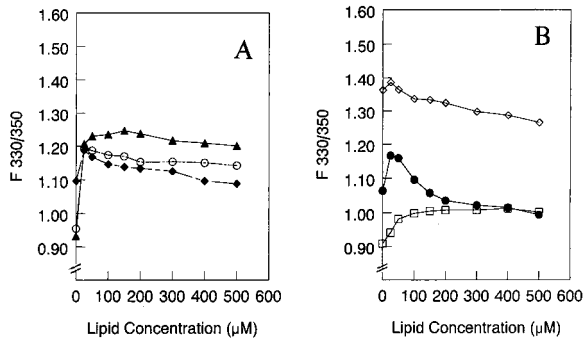

FIGURE 3: Binding of T domain to DOPC-containing model membranes at low pH. Samples with 0.33 μM T domain at pH 4 were titrated with small aliquots of 30%/70% DOPG/DOPC (mole/mole) SUV. (A) Fluorescence of wild-type T domain (○), W206 (▲), and W281 (♦). (B) Fluorescence of W355 (●), W364 (◇), and W375 (□).

Table 1: Fluorescence Quenching of Membrane-Associated Single-Trp T Domain Mutants by Spin-Labeled Phospholipids in DOPC-Containing Model Membrane Vesicles[a]

T domain	λ_{max} (nm)	F_{tc}/F_0	F_5/F_0	F_{12}/F_0	F_{12}/F_5
wild-type	336 ± 0.9	0.62 ± 0.01	0.59 ± 0.04	0.40 ± 0.03	0.67
W206	338 ± 1.2	0.70 ± 0.05	0.58 ± 0.05	0.44 ± 0.07	0.76
W281	338 ± 0.8	0.80 ± 0.06	0.70 ± 0.08	0.55 ± 0.07	0.78
W355	343 ± 0.9	0.59 ± 0.02	0.57 ± 0.04	0.53 ± 0.03	0.93
W364	334 ± 2.3	0.49 ± 0.06	0.41 ± 0.03	0.25 ± 0.05	0.60
W375	348 ± 0.6	0.43 ± 0.02	0.43 ± 0.02	0.47 ± 0.06	1.09

[a] Samples contained 0.33 μM T domain at a low protein-to-lipid ratio (protein-to-lipid molar ratio 1:1000) at pH 4. F_{tc}/F_0, F_5/F_0, and F_{12}/F_0 values are the ratios of fluorescence in the presence of 15% lipids carrying shallow (TempoPC), medium depth (5SLPC), and deep (12SLPC) nitroxides, respectively, to that in samples without the quencher. Mean values ± std deviation are shown. $F_{12}/F_5 = (F_{12}/F_0)/(F_5/F_0)$.

for the W355 mutant is consistent with this observation. The emission properties of this residue are consistent with it locating in a more nonpolar environment (e.g. inserts more deeply) at low lipid concentrations, where there is a high protein-to-lipid ratio, and then shifting to a more polar environment (e.g. inserts at a shallower depth) at high lipid concentrations, where there is a low protein-to-lipid ratio. That this reflects a dependence of the depth of insertion upon lipid-to-protein ratio is confirmed by quenching results (see below). The observation that W281 also shows a concentration-dependent fluorescence suggests that the protein-to-lipid ratio also may also result in conformational changes in a portion of the T domain other than the TH8/TH9 hairpin.

Trp Fluorescence Emission of Membrane-Inserted T Domain. The fluorescence emission of the different Trp residues was compared in more detail by measuring emission λ_{max} for the membrane-bound T domain. As shown in Tables 1−3 and Figure 4, under all conditions examined the order of λ_{max} was, from lowest to highest (i.e. from most blue-shifted to most red-shifted), W364 > W206,W281,W355 > W375. This order is consistent with residue 375 being in the most polar environment, residue 364 in the least polar environment, and the other residues insert in an environment of intermediate polarity.

For most residues, λ_{max} was not strongly affected by the T domain-to-lipid ratio or bilayer thickness (Tables 1−3 and Figure 4). However, as suggested by the lipid binding experiments described above, the emission of a Trp at residue

Table 2: Fluorescence Quenching of Membrane-Associated Single-Trp T Domain Mutants by Spin-Labeled Phospholipids in DMoPC-Containing Model Membrane Vesicles[a]

T domain	λ_{max} (nm)	F_{tc}/F_0	F_5/F_0	F_{12}/F_0	F_{12}/F_5
wild-type	335 ± 1.2	0.64 ± 0.02	0.61 ± 0.11	0.46 ± 0.01	0.75
W206	338 ± 1.4	0.70 ± 0.11	0.66 ± 0.11	0.53 ± 0.07	0.80
W281	337 ± 1.5	0.70 ± 0.06	0.68 ± 0.10	0.54 ± 0.01	0.79
W355	340 ± 0.8	0.72 ± 0.05	0.66 ± 0.04	0.50 ± 0.04	0.76
W364	336 ± 1.3	0.62 ± 0.08	0.63 ± 0.06	0.43 ± 0.06	0.68
W375	349 ± 0.3	0.48 ± 0.05	0.53 ± 0.06	0.52 ± 0.03	0.98

[a] Samples contained 0.33 μM T domain at a low protein-to-lipid ratio (protein-to-lipid molar ratio 1:1000) at pH 4.

Table 3: Fluorescence Quenching of Membrane-Associated Single-Trp T Domain Mutants by Spin-Labeled Phospholipids in DOPC-Containing Model Membranes Vesicles at a High Protein to Lipid Ratio[a]

T domain	λ_{max} (nm)	F_{tc}/F_0	F_5/F_0	F_{12}/F_0	F_{12}/F_5
wild-type	335 ± 1.2	0.64 ± 0.05	0.58 ± 0.01	0.55 ± 0.01	0.95
W206	338 ± 0.7	0.70 ± 0.05	0.62 ± 0.06	0.59 ± 0.07	0.96
W281	338 ± 0.9	0.85 ± 0.07	0.87 ± 0.09	0.74 ± 0.08	0.86
W355	339 ± 0.5	0.72 ± 0.03	0.76 ± 0.05	0.59 ± 0.04	0.77
W364	333 ± 3.0	0.78 ± 0.03	0.70 ± 0.09	0.53 ± 0.08	0.75
W375	347 ± 0.9	0.48 ± 0.02	0.49 ± 0.05	0.50 ± 0.03	1.02

[a] Samples contained 0.33 μM T domain at a protein-to-lipid ratio 1:150 at pH 4.

355 was strongly dependent on these factors. At low protein-to-lipid ratio in DOPC-containing vesicles the order of λ_{max} was W364 > W206 ≈ W281 > W355 > W375. However, W355 gave more blue-shifted fluorescence at a high protein-to-lipid ratio in DOPC-containing vesicles or when inserted into DMoPC-containing bilayers (Figure 4).[4] As a result the difference between λ_{max} of W355 and that of both W281 and W206 decreased. This is consistent with residue 355 inserting into a more nonpolar environment under the latter two conditions.

Depth of Trp Residues in DOPC-Containing Model Membranes at Low Protein-to-Lipid Ratio. Although the wavelength of maximum emission of a Trp is strongly affected by its depth within a membrane (*27*), emission data alone cannot reveal the depth of fluorescence groups within membranes, because they reflect a number of factors, such as the contributions of nearby residues to local polarity, the degree of solvent relaxation, and (unless corrected for) polarization artifacts. Therefore, the depth of Trp residues was examined more directly using fluorescence quenching with spin-labeled (nitroxide-labeled) lipids. To do this, the quenching of the Trp fluorescence of membrane-bound T domain by lipids carrying a shallow (TempoPC), medium (5SLPC), or deep (12SLPC) fluorescence quenching nitroxide group was measured. The quenching data are presented as the ratio of the fluorescence in the presence of the quencher to that without (F_0) quencher (100% fluorescence). The fraction of fluorescence unquenched in the presence of the shallow quencher is given by F_{tc}/F_0, by F_5/F_0 for the intermediate quencher, and by F_{12}/F_0 for the deep quencher. The relative levels of quenching were used to evaluate Trp depth (*27*, *28*). In particular the F_{12}/F_5 ratio was employed

[4] Because these values were obtained with an excitation wavelength of 295 nm (to eliminate Tyr fluorescence effects), the maximum emission intensity was slightly red-shifted relative to those in the lipid binding experiments in which the excitation wavelength was 280 nm.

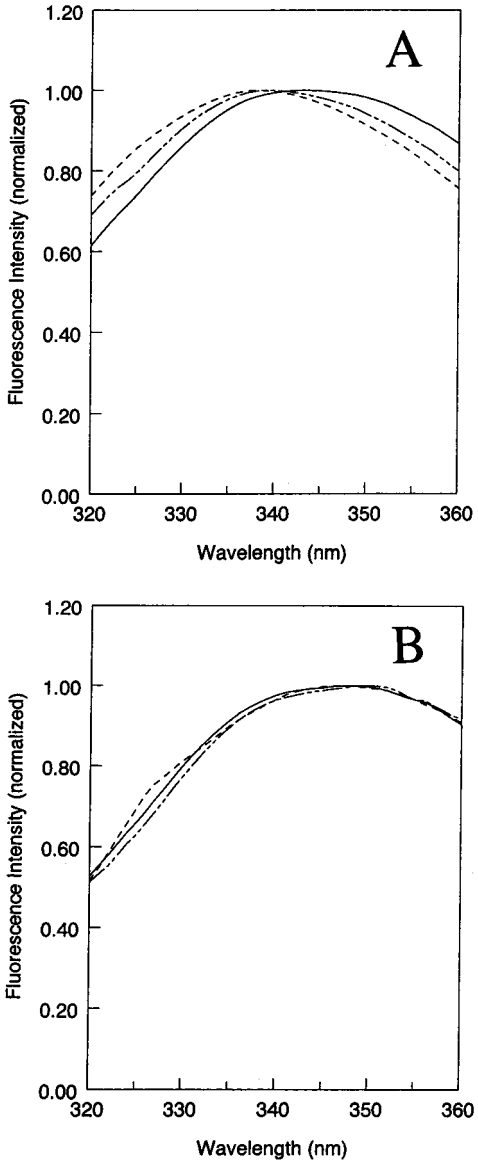

FIGURE 4: Trp fluorescence emission spectra of model membrane inserted T domain at low pH. (A) W355 emission spectra under the following conditions: (—) low protein-to-lipid ratio (protein to lipid molar ratio, 1:1000) in 30%/70% DOPG/DOPC vesicles; (— —) high protein-to-lipid ratio (protein to lipid molar ratio, 1:150) in 30%/70% DOPG/DOPC vesicles; and (— - - —) at a low protein-to-lipid ratio (protein to lipid molar ratio, 1:1000) in 30%/70% DOPG/DMoPC vesicles. (B) W375 emission spectra under the following conditions: (—) low protein-to-lipid ratio (protein to lipid molar ratio, 1:1000) in 30%/70% DOPG/DOPC vesicles; (— —) high protein-to-lipid ratio (protein to lipid molar ratio, 1:150) in 30%/70% DOPG/DOPG vesicles; and (— - - —) at a low protein-to-lipid ratio (protein to lipid molar ratio, 1:1000) in 30% DOPG/70%DMoPC vesicles.

to compare the difference in relative quenching between the intermediate and deep quenchers. A low F_{12}/F_5 value is indicative of relatively stronger quenching by 12SLPC as compared to 5SLPC, and thus of deep insertion.

Relatively deep insertion in DOPC-containing bilayers at a low protein-to-lipid ratio was found for the W206, W281, and W364 mutants (Table 1). In these cases the deep 12SLPC quencher exhibited the strongest quenching (i.e., the F_{12}/F_5 value was low). The behaviors of W206, which is in helix TH1, and W281, which is in helix TH5, suggest that at least some portions of TH1 and TH5 insert into the bilayer. W375 was found to be relatively shallowly located, (i.e., having the highest F_{12}/F_5 ratio) with the quenching by the 12SLPC slightly weaker than that by both of the more shallow quenchers. W355 was found to have an intermediate depth, with an intermediate F_{12}/F_5 value. Since W355, W364, and W375 are all in TH9, this suggests an orientation in which the C-terminus of TH9 protrudes from the bilayer.

It is noteworthy that the overall order of depth, from deepest to shallowest W364 > W206 = W281 > W355 > W375 is in good agreement with their degree of exposure to a nonpolar environment under the same conditions as judged by λ_{max} (see above).

Depth of Trp Residues in DMoPC-Containing Model Membranes. The depth of Trp residues was also examined in thinner DMoPC-containing model membranes (Table 2), in which previous studies on bimane-labeled T domain have indicated deeper TH9 insertion (*20*). In these vesicles, relatively deep insertion was again found for residues W206, W281, and W364 and shallow insertion for W375. The behavior of W206 and W281 indicates that helices TH1 and TH5 remain inserted in DMoPC-containing vesicles. It was also observed that W355 exhibited deeper insertion in DMoPC-containing bilayers relative to that in DOPC-containing bilayers, as shown by the decrease in F_{12}/F_5 ratio. This is in agreement with the blue shift in the emission wavelength of W355 in DMoPC-containing bilayers relative to that in DOPC-containing bilayers and confirms the reorientation of TH9 such that there is deeper insertion of the N-terminal region of the helix in DMoPC-containing vesicles (*20*).

Depth of Trp Residues in DOPC-Containing Model Membranes at a High Protein-to-Lipid Ratio. The fluorescence quenching patterns for the T domain were also examined in DOPC-containing model membranes under high protein-to-lipid ratio conditions (Table 3). For the residues in TH9, the patterns of Trp quenching were similar to those in DMoPC-containing bilayers. This suggests the presence of a deeply inserting conformation for TH9 similar to that in DMoPC and is in agreement with the similar λ_{max} for residue 355 in DMoPC-containing vesicles and at a high protein-to-lipid ratio in DOPC-containing vesicles. On the other hand, W206 appears to locate somewhat more shallowly in the bilayer (as shown by higher F_{12}/F_5) than in either DMoPC-containing or at lower protein-to-lipid ratios in DOPC-containing vesicles.

It should also be noted that at high protein-to-lipid ratios the overall level of quenching for all residues is slightly weaker (i.e., F/F_0 values are higher) relative to that at low protein-to-lipid ratios. This can also be seen in the quenching data for bimane-labeled T domain under conditions of high T domain concentration (*20*) and may be due to T domain oligomer formation, which could tend to reduce access of membrane embedded fluorescent groups to quencher-bearing lipids.

DISCUSSION

Conformation of Membrane-Inserted T Domain. The aim of this study was to use single-Trp mutants to define the conformations and topography of membrane-inserted T domain. One important conclusion arises from the observation that residues W206 and W281 insert into the membrane

bilayer. This indicates that a significant portion of both helices TH1 and TH5 must undergo membrane insertion. Such behavior is not unreasonable for helix TH5 because it is hydrophobic, composed solely of uncharged residues (*29*). In contrast TH1 is hydrophilic but has amphiphilic properties which could allow it to contact lipid on its more hydrophobic face, which contains W206 (*30*). In fact, a membrane spanning conformation for the TH1 region has been proposed on the basis of topography studies on cell-membrane-bound toxin (*31*).

The changes in the location of the Trp at position 355 observed at different T domain concentrations and bilayer widths are important because they confirm the existence of the two conformations we previously identified using labeled Cys mutants (*20*). These are conformations in which TH9 inserts shallowly (at low concentrations in DOPC-containing bilayers) and deeply (in thin DMoPC-containing bilayers and at high concentrations in DOPC-containing bilayers).

Interestingly, Trps at positions 206 and 281 are inserted under all the conditions examined, suggesting helices TH1 and TH5 are inserted in both the more shallowly and deeply membrane penetrating conformations. However, there was a small dependence of W281 fluorescence emission upon lipid-to-protein ratio observed in the lipid binding experiments and a small change in depth of W206 at high T domain concentrations, which may suggest that regions of the protein other than just the TH8/TH9 hairpin have a conformation dependent on protein concentration. Further studies with several mutants in various segments of the T domain sequence will be needed to define the nature of this conformational change.[5]

Schematic Models for T Domain Membrane Topography. Models of T domain helix orientation in a lipid bilayer based on the observations in this report are schematically depicted in Figure 5. Monomers are depicted in most cases, but it should be noted that oligomers may actually be present. Figure 5A illustrates a model for the T domain in its more shallowly inserted conformation, which is the conformation predominating at low protein-to-lipid ratios in DOPC-containing membranes. Because it was found that TH9 residues 355 and 364, but not 375, are inserted to a significant degree in this conformation, the TH8/TH9 hairpin is illustrated as tilted such that the N-terminal end of TH9 is more deeply embedded than the C-terminal end. Since residues 206 and 281 also insert into the bilayer, this model is drawn with the other helices of the T domain somewhat shallowly inserting into, or close to, the membrane surface. This model approximates the "penknife" model proposed for the hydrophobic helical hairpin of colicin channel-forming domains (*32*). However, we cannot rule out the possibility in which some the other helices (TH1−7) are transmembraneous under these conditions (also see below).

For the more deeply inserted conformation a number of possible structures also must be considered. On the basis of the results in this study, our previous study (*20*), and results on a series of Cys mutants (*33*), we propose TH8 and TH9

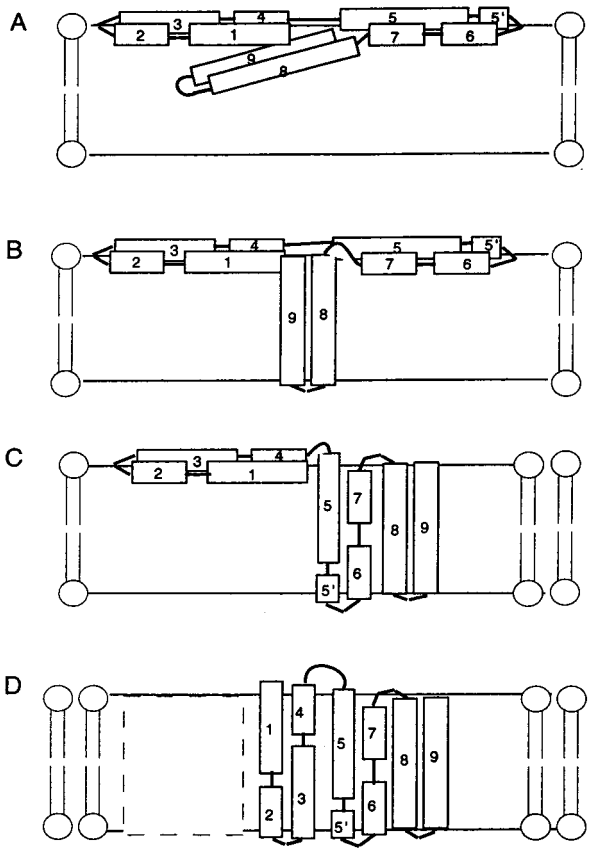

FIGURE 5: Schematic models for membrane-inserted T domain. (A) Model for the conformation in which TH9 is shallowly inserted. Not shown is the possibility that other helices are of transmembraneous orientation. (B−D) Models for the conformation in which TH9 is deeply inserted and in which various numbers of other helices exhibit deep insertion. In D the dotted outline represents a second T domain molecule that would be likely to be required to aid in burial of the more polar T domain helices. Oligomerization may also be present even for the other structures shown.

are transmembraneous in this conformation. One possibility is that the TH8/TH9 hairpin is the only transmembraneous portion of the T domain (Figure 5B). This would be similar to the "umbrella" model which has also been considered for the analogous helices in the colicin channel forming domain (*33*, *34*) and for the T domain (*9*).

It is also possible that there are other transmembrane helices (Figure 5C,D). Helices TH5−TH7 are relatively hydrophobic (*2*, *29*), and as noted above, TH1 has amphiphilic properties. Furthermore, even hydrophilic T domain helices could acquire a transmembrane orientation if shielded from the bilayer by other helices, as would be possible in an oligomeric structure. Topographic analysis of additional single site mutants in helices other than TH8 and TH9 will be necessary to distinguish between these different models.

Advantages and Limitations of Fluorescence Quenching Analysis of Residue Depth in Membrane Proteins. This study illustrates the utility of fluorescence quenching for examining the depth of individual Trp residues in a membrane protein within the bilayer and for identifying and characterizing different conformations by their effect on the depth of individual residues. We have introduced parallax analysis as a method for analyzing fluorescence quenching to obtain

[5] The effect of T domain concentration on Trp 281 emission was detected in the lipid binding experiments but not in the λ_{max} measurements. This may reflect a dependence on excitation wavelength which was 280 nm in the former experiments and 295 nm in the latter experiments.

[6] Kaiser, R., and London, E., unpublished results.

even more precise depths of fluorescent groups (*22*, *35*). It has been successfully applied to small molecules and helical polypeptides embedded in lipid bilayers (*22*, *26*, *35*−*40*). Nevertheless, we chose not to use parallax analysis in this study because fluorescence quenching is a function of how close a quencher can approach a fluorescent group laterally as well as the difference in depth of the quencher and fluorescent group. For a protein that partially penetrates the bilayer both the closest lateral approach and the difference in depth between quencher and a fluorescent residue are likely to depend on the depth of attachment of the quencher group to the lipid. In this case reliable *absolute* depths are difficult to obtain from fluorescence quenching.

Another consideration in evaluating depth values arises from the effect of the polarity of the fluorescent probe used. One goal of this study was to compare Trp as a probe to the bimane group we had used previously. As judged by the ratio of quenching by shallow and deep quenchers, it appears the depths obtained with Trp mutants were in general deeper than we previously observed using bimane-labeled mutants (*20*). This is likely to be due to the higher polarity of bimane relative to Trp.[6] Therefore, the effect of the fluorescence probes on apparent depth must also be considered.

One possible solution to these limitations is to use a systematic series of Trp or bimane-labeled Cys mutations and employ the pattern of relative changes in fluorescence quenching and other fluorescence properties to determine the topography of a membrane embedded segment. In this regard, results with a series of T domain mutants with single labeled Cys in TH8 and TH9 using methods other than nitroxide quenching give conclusions in good agreement with those in this study concerning the structure of TH9 under various conditions (*33*). This suggests the conclusions derived from nitroxide quenching cannot be seriously affected by perturbation of lipid structure due to the presence of the nitroxide probe on a fraction of the lipids.

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REFERENCES

1. Collier, R. J. (1982) in *ADP*−*Ribosylation Reactions: Biology and Medicine* (Hayashi, O., and Ueda, K., Eds.) pp 575−592, Academic Press, Inc., New York.
2. Choe, S., Bennett, M. J., Fujii, G., Curmi, P. M. G., Kantardjieff, K. A., Collier, R. J., and Eisenberg, D. (1992) *Nature 375*, 216−222.
3. Bennett, M. J., and Eisenberg, D. (1994) *Protein Sci. 3*, 1464−1475.
4. Bennett, M. J., Choe, S., and Eisenberg, D. (1994) *Protein Sci. 3*, 1444−1463.
5. Naglich, J. G., Metherall, J. E., Russell, D. W., and Eidels, L. (1992) *Cell 69*, 1051−1061.
6. Louie, G. V., Yang, W., Bowman, M. E., and Choe, S. (1997) *Mol. Cell 1*, 67−78.
7. London, E. (1992) *Biochim. Biophys. Acta 1113*, 25−51.
8. O'Keefe, D. O., Cabiaux, V., Choe, S., Eisenberg, D., and Collier, R. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6202−6206.
9. Zhan, H., Choe, S., Huynh, P. D., Finkelstein, A., Eisenberg, D., and Collier, R. J. (1994) *Biochemistry 33*, 11254−11263.
10. Silverman, J. A., Mindell, J. A., Zhan, H., Finkelstein, A., and Collier, R. J. (1994) *J. Membr. Biol. 137*, 17−28.
11. Mindell, J. A., Silverman, J. A., Collier, R. J., and Finkelstein, A. (1994) *J. Membr. Biol. 137*, 29−44.
12. Mindell, J. A., Silverman, J. A., Collier, R. J., and Finkelstein, A. (1994) *J. Membr. Biol. 137*, 45−57.
13. Mindell, J. A., Zhan, H., Huynh, P. D., Collier, R. J., and Finkelstein, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5272−5276.
14. Moskaug, J. O., Stenmark, H., and Olsnes, S. (1991) *J. Biol. Chem. 266*, 2652−2659.
15. Cabiaux, V., Quertenmont, P., Conrath, K., Brasseur, R., Capiau, C., and Ruysschaert, J.-M. (1994) *Mol. Microbiol. 11*, 43−50.
16. Quertenmont, P., Wattiez, R., Falmagne, P., Ruysschaert, J.-M., and Cabiaux, V. (1996) *Mol. Microbiol. 21*, 1283−1296.
17. Zhan, H., Oh, K. J., Shin, Y. K., Hubbell, W. H., and Collier, R. J. (1995) *Biochemistry 34*, 4856−4863.
18. Oh, K. J., Zhan, H., Cui, C., Hideg, K., Collier, R. J., and Hubbell, W. L. (1996) *Science 273*, 810−812.
19. Huynh, P. D., Cui, C., Zhan, H., Oh, K. J., Collier, R. J., and Finkelstein, A. (1997) *J. Gen. Physiol. 110*, 229−242.
20. Wang, Y., Malenbaum, S. E., Kachel, K., Zhan, H., Collier, R. J., and London, E. (1997) *J. Biol. Chem. 272*, 25091−25098.
21. Wang, Y., Kachel, K., Pablo, P., London, E. (1997) *Biochemistry 36*, 13600−13608.
22. Abrams, F. S., and London, E. (1993) *Biochemistry 32*, 10826−10831.
23. Blewitt, M. G., Chung, L. A., and London, E. (1985) *Biochemistry 24*, 5458−5464.
24. London, E. (1992) *Mol. Microbiol. 6*, 3277−3282.
25. Jiang, J. X., and London, E. (1990) *J. Biol. Chem. 265*, 636−8641.
26. Tortorella, D., Sesardic, D., Dawes, D. S., and London, E. (1995) *J. Biol. Chem. 270*, 27446−27452.
27. Ren, J., Lew, S., Wang, Z., and London, E. (1997) *Biochemistry 36*, 10213−10220.
28. Malenbaum, S. E., Merrill, A. R., and London, E. (1998) *J. Natural Toxins* (in press).
29. Greenfield, L., Bjorn, M. J., Horn, G., Fong, D., Buck, G. A., Collier, R. J., and Kaplan, D. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6853−6857.
30. vanderSpek, J. C., Howland, K., Friedman., and Murphy J. R. (1994) *Protein Eng. 7*, 985−989.
31. Madshus, I. H., Weidlocha, A., and Sandvig, K. (1994) *J. Biol. Chem. 269*, 4648−4652.
32. Lakey, J. H., Duche, D., Gonzalas-Manas, J.-M., Baty, D., and Pattus, F. (1993) *J. Mol. Biol. 230*, 1055−1067.
33. Kachel, K., Ren, J., Collier, R. J., and London, E. (1998) *J. Biol. Chem. 273*, 22950−22956.
34. Lakey, J. H., Baty, D., and Pattus, F. (1991) *J. Mol. Biol. 218*, 639−653.
35. Chattopadhyay, A., and London E. (1987) *Biochemistry 26*, 39−45.
36. Asuncion-Punzalan, E., Kachel, K., and London, E. (1998) *Biochemistry 37*, 4603−4611.
37. Chung, L. A., Lear, J. D., and DeGrado, W. F. (1992) *Biochemistry 31*, 6608−6616.
38. Jones, J. D., and Gierasch, L. M. (1994) *Biophys. J. 67*, 1534−1545.
39. Asuncion-Punzalan, E., and London, E. (1995) *Biochemistry 34*, 11460−11466.
40. Kaiser, R. D., and London, E. (1998) *Biochemistry 37*, 8180−8190.

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