Using a Novel Dual Fluorescence Quenching Assay for Measurement of Tryptophan Depth within Lipid Bilayers To Determine Hydrophobic α-Helix Locations within Membranes†

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ABSTRACT: A novel fluorescence method for determining the depth of Trp residues in membrane-inserted polypeptides is introduced. Quenching of Trp by acrylamide and 10-doxylnonadecane (10-DN) was used to measure Trp depth. Transmembrane helices with Trp residues at varying positions (and thus locating at different depths in lipid bilayers) were used to calibrate the method. It was found that acrylamide quenches Trp close to the bilayer surface more strongly than it quenches deeply buried Trp, while 10-DN quenches Trp close to the center of the bilayer more strongly than Trp close to the surface. The ratio of acrylamide quenching to that of 10-DN was found to be nearly linearly dependent on the depth of Trp in a membrane. It was also found that it was possible to detect coexisting shallowly and deeply inserted populations of Trp-containing polypeptides using these quenchers. In the presence of such mixed populations, acrylamide induced large blue shifts in fluorescence emission λ_{max} whereas 10-DN induced large red shifts. In a more homogeneous population quencher-induced shifts were found to be minimal. Dual quencher analysis can be used to distinguish hydrophobic helices with a transmembrane orientation from those located close to the bilayer surface and, when applied to a number of different peptides, revealed novel aspects of hydrophobic helix behavior.

A number of techniques, including diffraction, NMR, ESR, infrared spectroscopy, and fluorescence have been used to analyze membrane protein structure and behavior (*1*−*4*). Due to its sensitivity to environmental polarity (*5*), measuring the emission λ_{max} of Trp fluorescence has been one particularly useful tool for analyzing membrane protein structure and Trp depth. However, factors other than membrane depth can influence λ_{max} (*5*). Fluorescence quenching methods such as parallax analysis and distribution analysis can yield more precise values for Trp depth. These methods involve the comparison of the decrease in Trp fluorescence intensity induced by quenchers that are covalently attached to lipid molecules at different positions on their acyl chains or polar headgroup (*5*−*8*). Although very useful in a variety of systems, these methods can be difficult to apply to situations in which lipid structure (e.g., bilayer width) is varied. Methods using quenchers or analogous agents that are not located at fixed depths can be used in such systems, but their sensitivity to target depth must be calibrated empirically. A particularly successful example is an ESR method developed by Hubbell, Altenbach, and colleagues, in which the effects of aqueous and membrane-bound reagents that alter the ESR signal of spin-labeled amino acids are compared (*9*).

In this report we present a new fluorescence quenching method (dual quencher analysis, DQA)[1] that allows determination of the depth of Trp groups in lipid bilayers by using two quenchers that are not located at fixed depths: acrylamide and 10-doxylnonadecane (10-DN). Acrylamide is a widely used aqueous quencher of Trp that is unable to efficiently quench the fluorescence of Trp residues that are deeply buried in the core of a bilayer (*10*, *11*). The 10-DN molecule is a novel quencher of Trp fluorescence. It is a highly hydrophobic molecule containing a nitroxide-bearing doxyl group, a strong quencher of Trp fluorescence (*5*, *12*). The ratio of quenching by these probes was found to have an approximately linear dependence on Trp depth. Measurements of quenching ratio were made successfully in model membranes having different bilayer widths and lipid compositions. It was also found that quenching by acrylamide

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[1] Abbreviations: 10-DN, 10-doxylnonadecane; CPE, carboxypeptidase E peptide, $RKE_3KE_2LMEW_2KM_2SETLN$; DMoPC, diC14:1Δ9cPC (dimyristoleoyl-PC); DPoPC, diC16:1Δ9cPC (dipalmitoleoyl-PC); DOPC, diC18:1Δ9cPC (dioleoyl-PC); DEiPC, diC20:1Δ11cPC (dieicosenoyl-PC); DEuPC, diC22:1Δ13cPC (dierucoyl-PC); DNPC, diC24:1Δ15cPC (dinervonoyl-PC); DOPG, dioleoyl-*sn*-glycero-3-phosphoglycerol; DQA, dual quencher analysis; HPLC, high-performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NBD-PE, diC18:1Δ9c-*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-*sn*-glycero-3-phosphoethanolamine; PBS, 100 mM sodium phosphate and 150 mM NaCl, pH 7.1−7.3; pL(L11) = pL(W13), $K_2GL_9WL_9K_2A$; pL22, $K_2GL_{11}WL_{11}K_2A$; pL(P10P11), $K_2GL_6P_2LWL_9K_2A$; pL(D10D11), $K_2GL_6D_2LWL_9K_2A$; pL(W3), $K_2WL_9AL_9K_2A$; pL(W4), $K_2CWL_9AL_9K_2A$; pL(W5), $K_2CLWL_7AL_9K_2A$; pL(W9), $K_2CL_5WL_3AL_9K_2A$; pL(W11), $K_2CL_7WLAL_9K_2A$; pL(S11W13), $K_2GL_7SLWL_9K_2A$; SDSL, site-directed spin-labeling; 5-SLPC, 1-palmitoyl-2-(5-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; 12-SLPC, 1-palmitoyl-2-(12-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography.

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and 10-DN can detect heterogeneous peptide populations via their effects on Trp emission λ_{max}. Experiments using different membrane-inserted peptides show that DQA can answer fundamental questions about the behavior of membrane-inserted hydrophobic polypeptides.

EXPERIMENTAL PROCEDURES

Materials. Peptides $K_2GL_9WL_9K_2A$ [pL(L11) = pL(W13)], $K_2GL_{11}WL_{11}K_2A$ (pL22), $K_2GL_6PPLWL_9K_2A$ [pL(P10P11)], $K_2GL_6DDLWL_9K_2A$ [pL(D10D11)], $K_2WL_9AL_9K_2A$ [pL(W3)], $K_2CWL_9AL_9K_2A$ [pL(W4)], $K_2CLWL_7AL_9K_2A$ [pL(W5)], $K_2CL_5WL_3AL_9K_2A$ [pL(W9)], $K_2CL_7WLAL_9K_2A$ [pL(W11)], and $K_2GL_7SLWL_9K_2A$ [pL(S11W13)] were purchased from the Research Genetics Division of Invitrogen (Huntsville, AL). The N-termini of all of these peptides were acetylated, and the C-termini were amide-blocked. CPE peptide ($RKE_3KE_2LMEW_2KM_2SETLN$) was a gift from Y. Peng Loh (NIH). Phosphatidylcholines (1,2-diacyl-*sn*-glycero-3-phosphocholines) with even carbon number acyl chains in the series 14:1 to 24:1 (DMoPC, DPoPC, DOPC, DEiPC, DEuPC, and DNPC, respectively), DOPG, and NBD-PE were purchased from Avanti Polar Lipids (Alabaster, AL). Lipids were stored in chloroform at −20 °C. TLC revealed that the NBD-PE and DOPG each contained several percentages of a breakdown product that apparently formed during prolonged storage. The other lipids used were shown to be pure by TLC. Peptides were purified via reversed-phase HPLC using a C18 column with an 2-propanol/water/0.5% v/v trifluoroacetic acid gradient starting at 40% v/v 2-propanol (*5*, *13*). Peptide purity was checked using MALDI-TOF mass spectrometry (CASM, Stony Brook University) and was estimated to be above 90% in each case. Concentrations of purified peptides were measured by absorbance spectroscopy using a Beckman DU-650 spectrophotometer, using an ϵ for Trp of 5560 M^{-1} cm^{-1} at 280 nm. The hydrophobic peptides were stored at 4 °C in either 100% ethanol or 1:1 (v/v) 2-propanol/water. CPE was stored in 10 mM Tris-HCl and 150 mM NaCl, pH 8. Acrylamide was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution of 4 M acrylamide in water was used. 10-DN was purchased from Aldrich Chemical Co. (Milwaukee, WI). (Discontinued; contact authors for availability.) It was stored as a 2.7 mM stock solution in ethanol at −20 °C. Sepharose CL-4B was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

Model Membrane Vesicle Preparation. Model membranes were prepared using the ethanol dilution method (*5*). Peptides dissolved in ethanol or 1:1 2-propanol/water and lipids dissolved in chloroform were mixed and then dried under a stream of N_2. Samples were then dried under high vacuum for 1 h. To make ethanol dilution vesicles, 10 μL of 100% ethanol was added to dissolve the samples. Then 790 μL of PBS (100 mM sodium phosphate and 150 mM NaCl, pH 7.1−7.3) or of Tris−acetate buffer for CPE peptide (6.7 mM Tris-HCl, 150 mM NaCl, and 167 mM sodium acetate, pH 4.1) was added to the samples while vortexing to disperse the lipid−peptide mixtures. Unless otherwise specified, final concentrations were 2 μM peptide and 500 μM lipid.

Fluorescence Measurements. Fluorescence data were obtained on a SPEX τ2 fluorolog spectrofluorometer operating in the steady-state mode at room temperature. Measurements were taken on samples in semimicro quartz cuvettes (1 cm excitation path length and 4 mm emission path length) using a 2.5 mm excitation slit width and 5 mm emission slit width (band-pass of 4.5 and 9 nm, respectively). The excitation wavelength used to excite Trp was 280 nm unless otherwise noted. Fluorescence emission spectra were taken over the range 300−375 nm. Fluorescence from background samples containing lipid but no peptide was subtracted from reported values. Trp emission λ_{max} values in individual samples were generally within ±1 nm of the average values.

Acrylamide Quenching Measurements. To quantify acrylamide quenching, fluorescence was measured on samples containing model membrane-incorporated peptides or on background samples, both before and after the addition of a 50 μL aliquot of acrylamide from a 4 M stock solution dissolved in water. In these experiments fluorescence was measured at an excitation wavelength of 295 nm and an emission wavelength of 340 nm. This excitation wavelength was chosen to reduce acrylamide absorbance (and the resulting inner filter effect), and the emission wavelength was chosen to eliminate interference from the Raman band of water. Corrections were made both for dilution by the addition of acrylamide and for inner filter effect. Inner filter corrections were done by applying the formula $10^{\epsilon 0.5bC}$, where ϵ is the molar extinction coefficient at 295 nm in units of M^{-1} cm^{-1}, b is the path length of the cuvette in centimeters, and C is the concentration of acrylamide in the sample in molar (*14*).

10-Doxylnonadecane Quantification. The absorbance of 10-DN dissolved in ethanol was measured on a Beckman DU-650 spectrophotometer. Calculated molar extinction coefficients (ϵ) were 4480 M^{-1} cm^{-1} at the peak at 231 nm, 684 M^{-1} cm^{-1} at 280 nm, 14 M^{-1} cm^{-1} at 330 nm, and 12 M^{-1} cm^{-1} at the peak at 422 nm. Assuming these values are the same when 10-DN is incorporated in lipid bilayers, 10-DN would have only a negligible inner filter effect (less than 5%) at the concentrations used in the experiments (typically 20−60 μM), and so inner filter corrections were not made.

10-Doxylnonadecane Quenching Measurements. To measure the efficiency of 10-DN quenching, the fluorescence of samples in the absence of 10-DN was compared to that in its presence. Samples containing model membrane-incorporated peptides or background samples without peptide were prepared as noted above except that for the samples containing 10-DN either 10 mol % (for DOPC) or 12 mol % (for DEuPC) of the lipid was replaced by an equivalent amount of 10-DN. A larger fraction of quencher was used in the DEuPC samples to maintain a constant ratio of doxyl groups to hydrophobic volume [which is roughly 20% larger for DEuPC, which has C22:1 acyl chains, than it is for DOPC, which has C18:1 chains (*15*, *16*)]. This is appropriate because it appears that 10-DN molecules reside at a wide range of depths within the acyl chain region of the bilayer (see Results). By normalizing quencher concentration to hydrophobic volume, roughly the same number of quenchers should be present at each different depth in bilayers with different widths. For Trp fluorescence intensity measurements involving 10-DN samples were excited at 280 nm, and the emission intensity was measured at 330 nm.

Calculation of the Acrylamide to 10-DN Quenching Ratio (Q-Ratio). The ratio of quenching by acrylamide to that by 10-DN was used to estimate Trp depth in the membrane.

This quenching ratio (*Q*-ratio) was calculated from the formula *Q*-ratio = $[(F_o/F_{acrylamide}) - 1]/[(F_o/F_{10-DN}) - 1]$, where F_o is the fluorescence of the sample with no quencher present and $F_{acrylamide}$ and F_{10-DN} are the fluorescence intensities in the presence of acrylamide or 10-DN, respectively. This formula gave a more linear response to Trp depth than several other variations that were tested.

Measurement of Quencher-Induced Shifts in Emission λ_{max}. Samples containing either no quencher, acrylamide, or 10-DN were prepared as described above. Samples were excited at 280 nm and the emission spectra recorded over the range 300−375 nm. Emission λ_{max} values were obtained after background spectra were subtracted from samples lacking peptide. Similar results were found with an excitation wavelength of 295 and 280 nm. Despite a strong inner filter effect by acrylamide, the latter value was generally used because it gave more intense emission spectra and eliminated interference by the Raman peak of water.

Cobalt Quenching Experiments. Vesicle leakiness was monitored by Co^{2+} quenching of the fluorescence of (0.5 mol %) NBD-PE incorporated into DOPC vesicles (*17*). Vesicles were prepared by ethanol dilution as noted above except that a Tris buffer (10 mM Tris-HCl and 150 mM NaCl, pH 7.1) was used. To monitor leakiness, two samples were prepared. To the first sample 16 μL of 1 M $CoCl_2$ dissolved in water was added after vesicle formation. In this sample Co^{2+} should initially be only in the external aqueous solution. NBD fluorescence intensity was measured in this sample both before and approximately 5 min after $CoCl_2$ addition. In a second sample, 16 μL of 1 M $CoCl_2$ was premixed with the 790 μL of Tris buffer used to dilute the solutions of lipid dissolved in ethanol and initiate vesicle formation. As a result, in this sample Co^{2+} would be located both in the vesicle-entrapped and external aqueous solutions. For each case, background samples lacking NBD were also prepared. NBD fluorescence was measured at an excitation wavelength of 460 nm and an emission wavelength of 534 nm as described above. Fluorescence intensities from background samples lacking NBD-PE were subtracted for each sample. Fluorescence intensities were corrected for inner filter effects from the $CoCl_2$ (about 10%).

Assay of Acrylamide Permeability in Bilayers by Osmotic Shrinking. To determine whether acrylamide is able to cross bilayers rapidly, osmotic shrinking experiments were performed (*18*, *19*). Multilamellar vesicles composed of 95 mol % DEuPC/5 mol % DOPG were prepared at a concentration of 2 mM in 100-fold diluted PBS as described above for ethanol dilution vesicles, except that the addition of ethanol to the dried lipids just prior to addition of buffer was omitted. After the addition of buffer the samples were dispersed by vortexing for 1−2 min. Aliquots of 800 μL from the vesicle stock solution were introduced into a cuvette, and optical density (due to light scattering) was measured at 550 nm. After addition of 50 μL of water, 50 μL of 4 M acrylamide/water, or 35 μL of 5 M NaCl to different samples, and allowing optical density to stabilize, optical density was remeasured. In addition, an aliquot of 80 μL of 1.46 M sucrose was subsequently added to the sample to which water had been added, and optical density was remeasured after stabilization.

Effect of 10-DN upon λ_{max} of Trp Emission in Bilayers with Different Acyl Chain Lengths. Vesicles were prepared as noted above except that different 10-DN concentrations (as a mole fraction of lipid + 10-DN) were used, depending on the acyl chain length of the lipids that composed the bilayer. The amount of 10-DN used was such that the concentration of 10-DN molecules per unit hydrophobic volume would be relatively constant. A value of 10 mol % 10-DN was used in DOPC bilayers (which have 18 carbon long acyl chains). A larger fraction of quencher was used in the samples in which lipids had acyl chains longer than 18 carbons (DEiPC, DEuPC, DNPC) and a smaller fraction in those samples in which the lipid acyl chains were shorter than 18 carbons (DMoPC and DPoPC). The mole percent of 10-DN employed was calculated from the formula mol % 10-DN = (number of carbon atoms in lipid acyl chains/18) × 10%.

RESULTS

Quenching of Membrane-Embedded Trp Residues by Acrylamide. We wished to compare fluorescence quenching by a molecule that resides primarily in the aqueous solution to quenching by one located within a bilayer. Acrylamide was chosen as the aqueous quencher. First, the dependence of acrylamide-induced quenching upon Trp location within a lipid bilayer was examined. To do this, a series of Lys-flanked poly(Leu) peptides with a single Trp located at different positions in the primary sequence (and therefore at different depths in the bilayer) were incorporated into DOPC vesicles. These peptides form transmembrane helices in DOPC vesicles, and the depths of their Trp residues have been previously confirmed by parallax analysis (*5*).

As shown in Figure 1A acrylamide quenching [expressed by the ratio of fluorescence intensity in the absence of quencher (F_o) that in the presence of quencher (F)] was stronger for a Trp near the charged end of the helix than it was for one that is located at the center of the hydrophobic sequence. In addition, quenching was linearly dependent on acrylamide concentration. The quenching of the full series of Trp-containing peptides at a fixed acrylamide concentration is shown in Figure 1B. A nearly linear relationship between F_o/F and Trp position was observed for peptides incorporated into DOPC vesicles. This implies that F_o/F is also linearly related to Trp depth, which is linearly related to residue position in this system (*5*). Experiments with a peptide having a Trp at the bilayer center and one with a Trp close to the membrane surface showed that, in thicker bilayers (composed of DEuPC, a lipid which has 22:1 acyl chains), quenching by acrylamide was similar to that in DOPC vesicles. Similarly, acrylamide quenching of the Trp of these peptides in vesicles composed of a 1:1 mixture of DOPC and DOPG was also similar to that in DOPC vesicles. These experiments suggest that acrylamide quenching is relatively insensitive to these variations in lipid composition.

It should be noted that acrylamide has some solubility in organic solvents and should cross lipid bilayers rapidly (*36*). To demonstrate that acrylamide can cross wide bilayers, osmotic shrinking experiments were performed. In these experiments hypertonic concentrations of acrylamide or other solutes were added externally to multilamellar vesicles composed primarily of DEuPC. Externally added membrane-impermeable solutes induce an osmotic stress that causes osmotic shrinking of multilamellar vesicles and increased

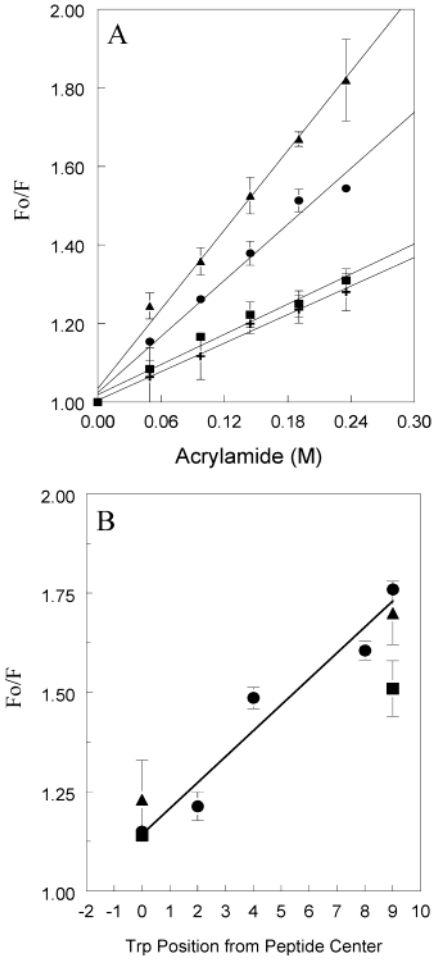

FIGURE 1: Acrylamide quenching of Trp fluorescence in transmembrane peptides in which the Trp position is varied. (A) Effect of acrylamide concentration upon quenching of 2 μM (crosses) pL-(W13), (squares) pL(W11), (circles) pL (W9), or (triangles) pL-(W4) incorporated into DOPC vesicles (200 μM lipid) dispersed in PBS. The *y*-axis shows the ratio of fluorescence intensity in the absence of quencher (F_o) to that in its presence (F). (B) Quenching at 235 mM acrylamide vs Trp position. Numbers on the *x*-axis give the position of Trp relative to the peptide center (which is at residue 13). Key: (circles) samples as for (A); (triangles) samples as for (A) except in vesicles composed of DEuPC; (squares) samples as for (A) except in vesicles composed of 1:1 DOPG/DOPC (mol/mol). A least-squares best-fit line is drawn through the DOPC values. Average values and standard deviations (except when smaller than symbol size) are shown for triplicates. Note that the pL(W4) peptide has one extra residue relative to the other pL peptides. Its Trp position is graphed as if the distance from N-terminal Lys determines depth.

optical density (*18*−*20*). Consistent with this behavior, hypertonic solutions of two membrane-impermeable molecules, NaCl and sucrose, induced osmotic shrinking (Table 1). However, addition of hypertonic acrylamide did not induce osmotic shrinking, even in the first few seconds after its addition, indicating that it crosses DEuPC bilayers rapidly (Table 1).

Quenching of Membrane-Embedded Trp by 10-DN. The molecule 10-DN was chosen as a membrane-embedded quencher. 10-DN is a derivative of the hydrocarbon nonadecane that contains a nitroxide-bearing doxyl group. Nitroxides are effective quenchers of Trp and other fluorophores (*5*, *19*). The dependence of the quenching of the fluorescence of transmembrane peptides with Trp in different positions upon 10-DN concentration is shown in Figure 2A. The exponential dependence of F_o/F upon 10-DN concentration was similar to that observed previously for nitroxide-labeled phospholipids (*19*). It reflects the fact that diffusion within bilayers is slow, so that quenching by an agent within a bilayer is dominated by the proximity of quenchers and fluorophores rather than by "collisional" events (*19*).

Table 1: Demonstration of Acrylamide Permeability by Detection of Osmotic Shrinking of Multilamellar Vesicles Containing 95% DEuPC/5% DOPG (mol/mol)

solute[a]	OD_{550}[b] prior to solute addition	OD_{550} after solute addition[c]
acrylamide[d]	0.70	0.68
NaCl[d]	0.71	2.11
sucrose	0.69	1.73
none (water)[d,e]	0.70	0.71

[a] Solutes were dissolved in water. The final concentration after addition to vesicles was 0.13 M for sucrose, 0.21 M for NaCl, and 0.23 M for acrylamide. [b] OD = optical density. [c] Readings after stabilization of OD (1−2 min). [d] Control samples containing 10 μL of ethanol in addition to acrylamide gave similar results. [e] In the mock control, water not containing solute was added.

When the quenching of fluorescence for a full series of transmembrane peptides with varying Trp positions by 10 mol % 10-DN was measured in DOPC-containing vesicles, a nearly linear dependence of F_o/F upon depth was observed (Figure 2B). The dependence of F_o/F on Trp depth was the inverse of that observed for acrylamide, with a deeply located Trp being more strongly quenched than a shallow one. Similar results were observed with 5 mol % 10-DN, except that, as expected, quenching was not as strong.

Experiments with a peptide having a Trp at the bilayer center and one with a Trp close to the membrane surface showed that in thicker bilayers (composed of DEuPC, a lipid which has 22:1 acyl chains) quenching by 10-DN was similar to that in DOPC vesicles. Similarly, 10-DN quenching of the Trp of these peptides in bilayers composed of a 1:1 mixture of DOPC and DOPG was also similar to that in DOPC vesicles. These experiments suggest that, like acrylamide quenching, 10-DN quenching is relatively insensitive to these variations in lipid composition.

To further characterize 10-DN behavior, its quenching of Trp was compared to that by lipids containing nitroxide groups at "fixed" depths. As shown in Table 2, the pattern of 10-DN quenching as a function of Trp depth falls between that previously found for phospholipids with nitroxide groups attached to the 5-carbon (5-SLPC) and 12-carbon (12-SLPC) of one acyl chain (*5*). 5-SLPC quenches shallow Trp more strongly than deep Trp, and 12-SLPC quenches deep Trp more strongly than shallow Trp. Although, like 12-SLPC, 10-DN quenches deep Trp more strongly than shallow Trp, 10-DN quenching is much more weakly dependent on Trp depth than that of 12-SLPC. The weak dependence of 10-DN quenching on Trp depth suggests that the nitroxide group of 10-DN is distributed over a wide range of depths within the hydrophobic core of the bilayer. In other words, the free energy of 10-DN molecules with the doxyl group at various depths must be similar. This is consistent with the hydrophobic character of the 10-DN molecule, which should not force it to be anchored at a specific depth.

To ascertain whether dissociation of 10-DN from vesicles would affect quenching measurements, the effect of diluting 10-DN-containing vesicles upon quenching was measured.

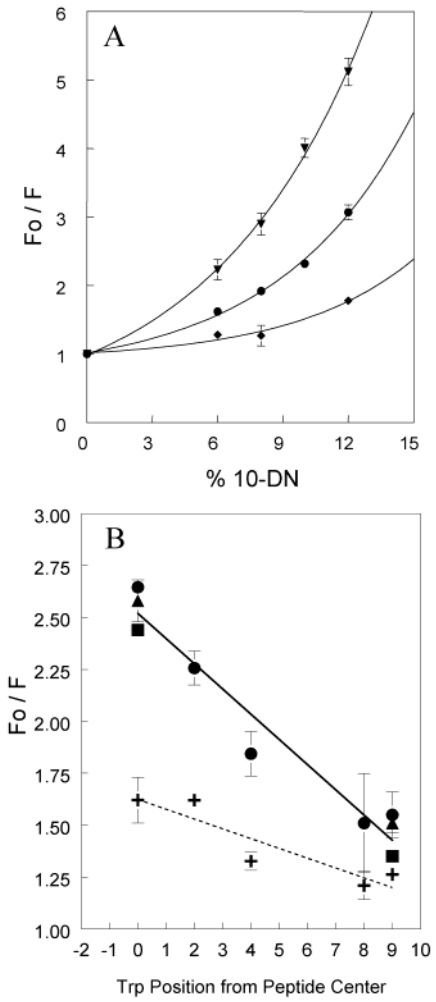

FIGURE 2: 10-DN quenching of Trp fluorescence in transmembrane peptides in which the Trp position is varied. (A) Effect of 10-DN concentration (as a mole percent of lipid plus 10-DN) upon quenching of 2 μM (triangles) pL(S11W13), (circles) pL (W9), or (diamonds) pL(W5) incorporated into DOPC vesicles (200 μM lipid) dispersed in PBS. (B) Quenching by 10-DN vs Trp position. Numbers on the *x*-axis give the position of Trp relative to the peptide center. Key: (circles) samples as in Figure 1A except with 10 mol % 10-DN in place of acrylamide; (triangles) samples as for Figure 1A except in vesicles composed of DEuPC with 12 mol % 10-DN; (squares) samples as for Figure 1A except in vesicles composed of 1:1 DOPG/DOPC (mol/mol) with 10 mol % 10-DN; (crosses) samples as in Figure 1A except with 5 mol % 10-DN in place of acrylamide. Average values and standard deviations (except when smaller than the symbol size) are shown for triplicates. A least-squares best-fit line is drawn through the values for the DOPC samples containing either 5 or 10 mol % 10-DN.

The quenching of the fluorescence of a membrane-incorporated poly(Leu) peptide [pL(L11)] only decreased slightly (from 69% quenching to 65% quenching) upon 8-fold dilution of a sample with buffer (from 500 to 62.5 μM lipid). Extended incubation at the diluted concentration (10 h) had no additional effect upon quenching (64% quenching). These experiments indicate that 10-DN is strongly bound to lipid bilayers and that few 10-DN molecules dissociate from bilayers even at very low lipid concentrations.

Effect of 10-DN on Vesicle Properties. Membrane perturbations due to the presence of 10-DN could affect interpretation of quenching data. Therefore, the effects of 10-DN on vesicle size, bilayer width, and vesicle leakiness were studied. Size exclusion chromatography on Sepharose 4B-CL, as well as dynamic light scattering, indicated that, at most, there were only very slight differences in vesicle size between samples lacking and containing 10 mol % 10-DN (data not shown).

Table 2: Comparison of Quenching of Trp at Different Positions in Transmembrane Peptides by Lipids Carrying Nitroxides at Fixed Locations within the Bilayer to Quenching by 10-Doxylnonadecane

	F/F_o[a]		
peptide	5-SLPC[b]	12-SLPC[b]	10-DN
pL(W3)[c]	0.32	0.41	0.645[d]
pL(W5)	0.325	0.335	0.662
pL(W9)	0.355	0.16	0.542
pL(W11)	0.56	0.105	0.443
pL(W13)	0.59	0.041	0.378

[a] F/F_o is the ratio of Trp fluorescence intensity in DOPC-containing vesicles containing quencher to that of vesicles without quencher. [b] 5-SLPC and 12-SLPC are the quencher lipids 1-palmitoyl-2-(5- or 12-doxyl)stearoylphosphatidylcholine, respectively. Data shown for these quenchers comes from Ren et al. (*5*). For 5- and 12-SLPC the quencher concentration was 15 mol % whereas the 10-DN was used at 10 mol %. [c] The Trp residue position relative to the N-terminus is given by number after W. [d] The pL(W4) peptide was used in place of the pL(W3) peptide for 10-DN experiments.

Cobalt quenching of NBD attached to lipid headgroups was used to test the effect of 10-DN on vesicle integrity/leakiness. Vesicles were prepared with a trace amount of NBD-PE. In one experiment $CoCl_2$ was added externally to samples with or without 10-DN. Because Co^{2+} is unable to cross bilayers rapidly (*21*), only the NBD groups in the outer monolayer of the bilayer should be quenched in these samples. In a second set of samples $CoCl_2$ was present in the buffer added to form the vesicles, so that Co^{2+} was both in the external solution and in the vesicle lumen, and thus able to quench NBD groups in both the inner and outer monolayers. If 10-DN caused bilayers to become leaky, quenching should have been independent of the preparation procedure, whereas in the absence of a leak there should have been considerably less quenching with Co^{2+} initially restricted to the external solution. In both the absence and presence of 10-DN initially restricting Co^{2+} to the external solution gave 2-fold less quenching than that observed with Co^{2+} on both sides of the bilayer (Figure 3A). No evidence for Co^{2+} leakage was seen when incubation times in 10-DN-containing samples were extended from 5 to 90 min (data not shown). These experiments show that in both the absence and presence of 10-DN vesicles are not leaky to Co^{2+}. Incorporation of a hydrophobic peptide [pLeu(D10D11)] within the bilayer also had no effect on Co^{2+} quenching (data not shown), indicating that such peptides are not likely to be forming aqueous pores which could affect the interpretation of quenching data (see Discussion).

10-DN is a derivative of an aliphatic hydrocarbon, and hydrocarbon addition can increase bilayer width (*5*, *15*, *22*). If 10-DN altered bilayer width significantly, it would be of concern because width influences the behavior of transmembrane peptides (*5*, *23*−*25*). To examine whether 10-DN alters bilayer width, the effect of acyl chain length on peptide behavior was measured in both the presence and absence of 10-DN. To assess peptide behavior both the Trp λ_{max} and the slightly more sensitive ratio of Trp emission intensity at 350 nm to that at 330 nm (*26*) were monitored for the poly(Leu) peptide pL22. The Trp of pL22 has a fluorescence emission that is sensitive to bilayer width (*23*). As shown in

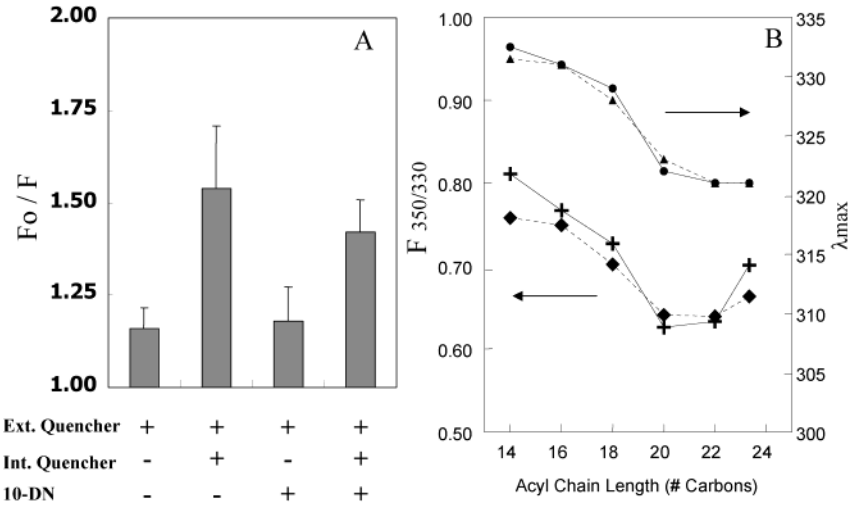

FIGURE 3: Tests for perturbation of vesicle integrity or bilayer width by 10-DN. (A) Effects of 10-DN on vesicle leakiness assayed by Co^{2+} quenching of NBD-PE. Samples contained 0.5 mol % NBD lipid incorporated into DOPC vesicles (200 μM lipid) with or without 10 mol % 10-DN dispersed in 10 mM/150 mM Tris-HCl/NaCl, pH 7.1. Quenching was induced by the presence of 20 mM $CoCl_2$ in either the external (ext) or both internal (int) and external solution. F_o/F is the ratio of NBD fluorescence in the absence of Co^{2+} to that it its presence. **(**B) Effect of 10-DN on emission spectra as a function of bilayer width: (crosses) the ratio of emission intensity at 350 nm to that at 330 nm ($F_{350/330}$) without 10-DN; (diamonds) $F_{350/330}$ with 10-DN; (circles) λ_{max} without 10-DN; (triangles) λ_{max} with 10-DN. Samples contained 2 μM pL22 peptide in 200 μM lipid plus 10-DN molecules dispersed in PBS. The concentration of 10-DN was 10 mol % in DOPC vesicles and normalized to give an equal number of 10-DN to hydrophobic volume for the other lipids used (see Experimental Procedures). Trp emission λ_{max} values in individual samples were generally within $\pm$1 nm of the average values.

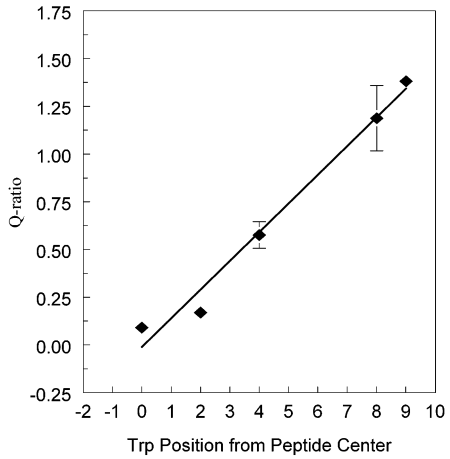

FIGURE 4: Quenching ratio (*Q*-ratio) vs Trp depth. The ratio of quenching by acrylamide to that by 10-DN [$(F_o/F_{acrylamide} - 1)/(F_o/F_{10-DN} - 1)$] vs Trp position is shown. Quenching data are from Figures 1B and 2B. Average values and standard deviations for triplicates are shown.

Figure 3B, addition of 10-DN to vesicles generally had little effect on Trp emission wavelength, indicating that 10-DN has relatively little effect on bilayer width.

Ratio of Acrylamide Quenching to 10-DN Quenching and Application of Quenching to Poly(Leu) Peptides with Different Sequences. Measuring quenching by a single quencher can yield information on depth, but measuring the ratio of quenching by two quenchers can amplify sensitivity and cancel out any non-depth-related effects on quenching (see Discussion). Therefore, the relationship of Trp depth to the ratio of quenching by acrylamide to that by 10-DN (*Q*-ratio) was calculated using the above-described quenching data for membrane-inserted poly(Leu) peptides. Like quenching by the individual quenchers, the *Q*-ratio showed a relatively linear dependence upon Trp depth in the bilayer (Figure 4). Trp near the bilayer center gave a *Q*-ratio in the range 0.1−0.2 whereas a Trp at the polar/nonpolar interface gave a value close to 1.5.

To see if the *Q*-ratio obtained by this dual quencher analysis (DQA) was sensitive to peptide sequence and behavior, it was applied to membrane-associated peptides with various sequences and properties. The pL(L11) peptide was used as an example of a transmembrane poly(Leu) peptide with a Trp at the center of its hydrophobic sequence (*5*, *23*). To examine the behavior of a peptide that should form a less stable transmembrane structure, the method was also applied to an analogous poly(Leu) peptide with two consecutive proline residues replacing leucine residues near the center of the hydrophobic core [pL(P10P11)]. In addition, the method was applied to the CPE peptide, derived from a membrane-associating segment of a carboxypeptidase (*27*). The CPE peptide has two consecutive Trp near the center of the peptide sequence. It was chosen as a representative peptide that, despite a strong association with membranes, is not highly hydrophobic and does not form a transmembrane structure (*27*). Finally, the behavior of pL22, a poly(Leu) peptide analogous to pL(L11) but with a longer Leu-rich sequence, was examined. The pL22 peptide was chosen because it tends to form oligomers within bilayers (*23*).

Figure 5 shows acrylamide quenching values, 10-DN quenching values, and *Q*-ratios for these peptides. As expected, the pL(L11) peptide has a Trp that locates at or very close to the bilayer center in DOPC vesicles (*Q*-ratio < 0.1). This is also true for pL(L11) in vesicles composed of DEuPC (*Q*-ratio about 0.1; see acrylamide and 10-DN quenching data in Figures 1B and 2B). As described in previous reports, when a peptide having a Trp at the center of the hydrophobic sequence and charged helix-flanking residues is found to have its Trp located at the center of the bilayer, it is a strong indicator of a stable transmembrane orientation (*5*, *23*). Therefore, the *Q*-ratios for pL(L11) are consistent with a transmembrane orientation in both DOPC and DEuPC vesicles.

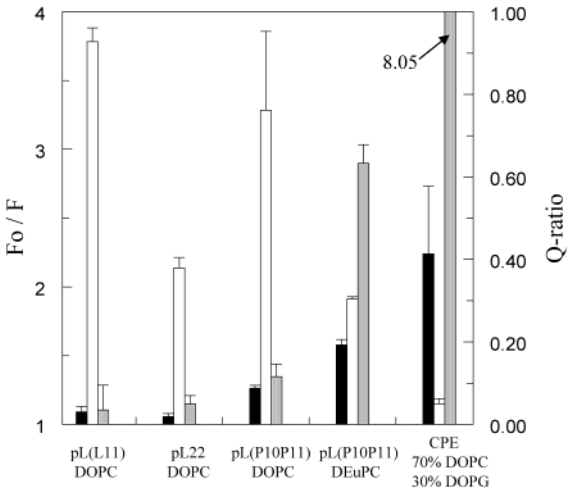

FIGURE 5: Effect of amino acid sequence upon acrylamide and 10-DN quenching of peptides incorporated into lipid vesicles: (black bar) F_o/F for acrylamide quenching; (open bar) F_o/F for 10-DN quenching; (gray bar) *Q*-ratio. Samples contained 2 μM peptide in vesicles composed of 200 μM lipid dispersed in PBS (except in the case of the CPE peptide-containing samples which were prepared in Tris−acetate buffer, pH 4.1). Average values and standard deviations for triplicates are shown. The standard deviation for the off-scale *Q*-ratio for CPE is ±1.8.

Quenching of the pL(P10P11) peptide incorporated into DOPC vesicles also showed a Trp location near the bilayer center (*Q*-ratio = 0.12). This shows that a transmembrane orientation can be maintained even in the presence of two Pro residues. However, in the wider DEuPC bilayer the Trp residue of this peptide appeared to be significantly more shallowly located, giving a *Q*-ratio (0.65) that corresponds to an intermediate depth in the bilayer rather than a location at the bilayer center. As shown below, this results from the presence of a mixture of molecules with different Trp depths. A fuller consideration of the behavior of this peptide, and of a series of poly(Leu) peptides with various different polar substitutions, is described in the accompanying report (*28*).

In contrast to the poly(Leu) peptides studied above, the bilayer-bound CPE peptide showed a very shallow Trp depth in DOPC-containing bilayers (*Q*-ratio = 8.05). This location is consistent with its highly red shifted fluorescence (*27*). In fact, the two Trp of CPE located more closely to aqueous solution than a Trp at the interface between the polar and nonpolar portions of the bilayer (which gives a *Q*-ratio close to 1.5; see above). This suggests that, even when membrane-bound, the sequence of a peptide can overcome the known preference of Trp residues for locating at the polar−nonpolar interface (*12*, *29*). It also implies that the center segment of the CPE peptide (which is its most hydrophobic section as judged by the Kyte−Doolittle scale; not shown) does not penetrate deeply into bilayers when membrane-bound.

When incorporated into DOPC vesicles, the oligomer-forming pL22 peptide gave a low *Q*-ratio similar to that for pL(L11), indicating it also has a Trp location at the bilayer center (Figure 5). This is in agreement with previous measurements of Trp depth for pL22 using parallax analysis of quenching by nitroxide-labeled lipids (*23*) and implies that oligomerization does not interfere with DQA. Figure 5 also shows that the absolute levels of both acrylamide and 10-DN quenching of pL22 were significantly lower than those for pL(L11). This is consistent with a reduction in quenching due to steric interference, arising from the burial of the Trp within pL22 oligomers and/or with decreased sensitivity to quenching due to a shortened Trp excited-state lifetime in pL22 oligomers. In either case, this behavior shows how factors that distort the depth dependence of quenching by *individual* quenchers are canceled out by using the ratio of quenching values with dual quenchers.

Detection of Multiple Populations of Trp at Different Depths Using Quencher-Induced λ_{max} Shifts. In the course of these experiments it was found that use of dual quenchers allows detection of heterogeneous peptide populations through their effect on Trp λ_{max}. Quenchers affect Trp λ_{max} because of its correlation with depth. In the absence of quenchers the emission spectrum of a sample containing both deep and surface-located Trp residues would be a composite of a blue-shifted spectrum and a red-shifted spectrum, respectively. Trp inhabiting the surface would be more sensitive to quenching by acrylamide, whereas the deep Trp would be more sensitive to quenching by 10-DN. The result should be a blue shift of Trp λ_{max} in the presence of acrylamide and a red shift in the presence of 10-DN. In contrast, no quenching-induced shift in λ_{max} should be observed for a uniform population in which all of the Trp residues have the same depth in the bilayer and same emission λ_{max}. However, even for a relatively uniform population small quenching-induced shifts are expected due to the distribution of peptides around an average depth (*5*, *23*).

A demonstration of the sensitivity of Trp λ_{max} to quenchers and peptide behavior in DEuPC bilayers is shown in Figure 6. The Trp emission spectra of the deeply located Trp of pL(L11) showed only small differences between λ_{max} (2 nm) with and without quenchers (Figure 6A), indicating a relatively uniform population in terms of depth. In contrast, emission spectra of the Trp of pL(P10P11), which has an intermediate average depth, showed large (8−9 nm) λ_{max} shifts in the presence of quenchers (Figure 6B). These shifts were in the directions predicted above for acrylamide and 10-DN quenching. Thus, in DEuPC bilayers the pL(P10P11) peptide formed a mixture in which some Trp had very blue shifted fluorescence, presumably arising from a transmembrane population of peptides in which the Trp is near the center of the bilayer, while other Trp had highly red shifted fluorescence, presumably arising from peptides located in the polar environment at the surface of the bilayer (see ref *28* for details). This latter population is likely to be bound to the bilayers, as demonstrated by the strong bilayer association of an even more polar peptide (*28*).

The observation that acrylamide and 10-DN induce shifts in the emission spectrum of pL(P10P11) to a similar extent indicates that deeply and shallowly located peptides both contribute strongly to pL(P10P11) fluorescence in the absence of quencher. If one population had been predominant in terms of fluorescence intensity, the quencher to which the predominant population was most susceptible would have likely induced a much larger shift than the other quencher. Quantitative analysis of such effects is complex (see Discussion).

Inspection of the spectra in Figure 6 also shows that DEuPC-incorporated pL(P10P11) has a much broader emission spectrum in the absence of quencher than does DEuPC-incorporated pL(L11). Because composite spectra formed by the superposition of spectra with different emission maxima

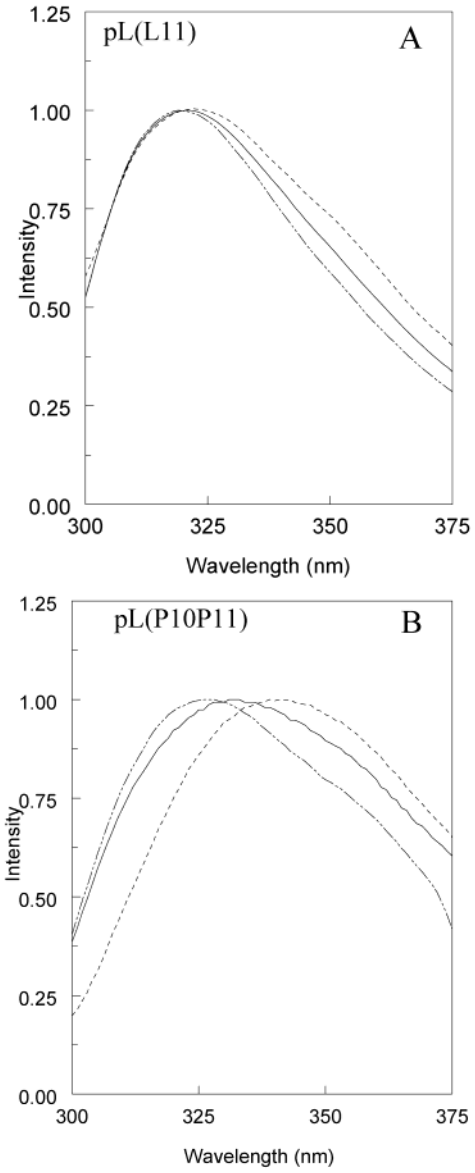

FIGURE 6: Effect of quenchers upon Trp spectra of hydrophobic peptides incorporated into lipid vesicles: (A) pL(L11) peptide; (B) pL(P10P11) peptide. Spectra are shown for 2 μM peptide incorporated into 200 μM DEuPC bilayers dispersed in PBS in the absence of quencher (solid lines), in the presence of 235 mM acrylamide (long and short dashes), or in the presence of 12 mol % 10-DN (short dashes). Intensities are normalized to 1.00 at λ_{max}.

are broadened (*5*), this is consistent with the quenching data showing that pL(P10P11) forms multiple populations.

DISCUSSION

Advantages and Disadvantages of the Dual Quencher Analysis (DQA) Relative to Other Fluorescence Quenching Assays of Trp Depth. Fluorescence quenching methods are widely used in studies of membrane structure but can be controversial if their accuracy and reliability are not clearly established (*8*, *30*). The calibration and controls described in this report show that the DQA method allows for relatively quick and unambiguous determination of the depth of Trp residues in model membranes.

Since there are other methods that can determine depth from fluorescence quenching experiments, it is important to compare the strengths and weaknesses of different approaches. One alternate approach is to determine depth with a single quencher that locates close to the bilayer center. If fluorescent substitutions spanning a range of amino acid positions are prepared, the center and ends of a transmembrane segment can be located by identifying the fluorescently labeled residues that are the most weakly and strongly quenched (*8*, *31*). This powerful approach requires fluorescent groups all along the sequence in question. It can potentially be confounded when the vulnerability of a fluorescent group to quenching is complicated by factors other than depth, such as differing locations relative to the lipid−protein interface or a dependence of its excited -state lifetime upon local environment. Relative to single quencher approaches, DQA has an advantage because it combines quenching results from two different quenchers, which tends to cancel out factors influencing quenching other than depth.[2] In addition, use of the ratio of quenching by two independent quenchers amplifies the sensitivity of DQA to depth. Finally, only a single fluorescent group is needed for DQA.

DQA also has some advantages over other fluorescence quenching assays using multiple quenchers, i.e., parallax analysis (PA) and distribution analysis (DA) (*5*, *8*). Because DQA uses quenchers with very different locations, it yields differences in quenching that are much larger and more easily measured than those obtained with PA and DA, methods using quenchers placed at only slightly different depths in the bilayer. A consequence of the increased difference in quenching is that DQA does not require as precise a calibration of quencher concentrations as does PA or DA. Another advantage of DQA relative to these methods is its adaptability to a wider range of lipid compositions. In PA and DA the nitroxide and brominated lipids commercially available are derivatives of PCs and comprise a significant fraction of the total lipid (up to 100% for brominated lipids). DQA avoids the need to synthesize new quenchers for studies of bilayers composed of lipids other than PCs.

On the other hand, unlike PA and DA, obtaining a precise value for depth from DQA requires an empirical calibration curve with a series of fluorophores at known depths and, thus, at present can only be used for Trp. In addition, it is possible that interpretation of DQA data may be complicated for Trp residues deeply buried within a protein. The exact extent to which 10-DN and acrylamide quenching is reduced when a Trp is located in the protein interior may not be identical if acrylamide approaches a Trp from the membrane surface[3] while 10-DN approaches it through the bilayer interior. However, the quenching of the oligomeric pL22 peptides suggests that *Q*-ratios will not be strongly influenced by this factor. Nevertheless, differences in the way quenchers approach a polypeptide would be of concern in the extreme case of a pore-forming protein in which the pore is accessible to acrylamide. DQA might measure distance from the pore rather than distance from the bilayer surface for such proteins. This is not a concern for this study and for the accompanying

[2] 10-DN quenching may not have exactly the same dependence on excited-state lifetime as does acrylamide quenching, so the cancellation of lifetime effects may not be complete.

[3] This is further complicated by the fact that acrylamide has some solubility in organic solvents, suggesting that some acrylamide molecules reside in the bilayer. It is not clear whether acrylamide quenching is only due to the acrylamide molecules in aqueous solution or whether those in the bilayer quench to a significant degree.

report (*28*) because the peptides used are unlikely to be pore-forming molecules (see Results). Nevertheless, it is important to note that the effects of fluorophore burial within a protein, or the presence of an aqueous pore, is much less of a problem when quenchers used are all restricted to the bilayer, as in PA and especially DA (*32*).

The results of this study suggest that perturbation of vesicle structure by 10-DN is not of great concern. It should be noted that, with its 19 carbon long hydrocarbon chain, 10-DN should occupy about the volume of one acyl chain, not two. Thus 10 mol % 10-DN should increase the volume of the hydrophobic part of the bilayer by only 5%. If the 10-DN all accumulated at the bilayer center, this would result in an increase in thickness equivalent to 1 carbon atom per leaflet. This is like changing from an 18 to a 19 carbon acyl chain. In fact, the effect of 10-DN on width is likely to be very small because 10-DN resides at various depths, not at the bilayer center (see Results). For experiments that are very sensitive to membrane perturbation it should be noted that DQA can be used with 5 mol % 10-DN, although quenching will be weaker and more difficult to measure accurately.

A variation of the DQA method could be developed using a doxyl fatty acid in place of 10-DN. This type of quencher was not chosen because of the relatively weak binding of doxyl fatty acids to bilayers, which necessitates difficult corrections for unbound quencher (*33*), and because of the perturbing effects of charged fatty acid carboxyl groups (*34*).

Detection of Multiple Populations of Fluorophores at Different Depths. A particularly useful feature of DQA is its ability to readily detect multiple fluorophore populations via quencher-induced λ_{max} shifts. This arises from relatively selective quenching of shallow or deep Trp populations by acrylamide or 10-DN, respectively. When multiple populations of Trp are present, DQA could in theory also be used to estimate the λ_{max} values of each population (see Results). Ideally, this should be done with enough quencher to completely quench one of the Trp populations. However, complete quenching of one population can never be achieved. Instead, quencher-shifted spectra will tend to give lower or upper limits to the λ_{max} of the most quencher-resistant population. To estimate the λ_{max} of an individual population more precisely, it may be necessary to measure λ_{max} as a function of quencher concentration and then extrapolate to infinite quencher concentrations. The relatively selective quenching of one population needed for this procedure to work is likely to be especially difficult for Trp populations that differ only slightly in depth.

It should also be noted that wavelength shift experiments can in principle yield information about the relative fraction of fluorescence arising from each population prior to quencher addition. If there are large populations of both shallowly and deeply located Trp, then both 10-DN and acrylamide quenching should induce large shifts. If the shallow population predominates, then strong acrylamide quenching should give a larger shift than 10-DN quenching, whereas if the deep population predominates, then strong 10-DN quenching should give the larger shifts. However, quantitative analysis of such effects would be complicated by factors such as spectral shape and how strongly fluorescence is quenched.

It should be noted that it is also possible to detect of multiple fluorophore populations by PA and DA. Under favorable conditions, the exact depth of each population can be established by DA (*35*). However, this requires a larger number of quenchers (minimally, three for PA and four for DA) as well as favorable fluorophore depth distributions.

Comparison of DQA to the ESR Analysis of Site-Directed Spin-Labeling (SDSL). The DQA method is analogous to the ESR-based SDSL analysis (*9*). Therefore, it is not surprising that in many respects SDSL and DQA share the same advantages and disadvantages relative to other methods. Furthermore, in terms of perturbations induced by the signal-generating probe, a Trp residue and small spin-label used in SDSL analysis are likely to be similar.

Nevertheless, DQA and SDSL analyses do differ in important ways. Oxygen, used as the membrane-bound signal-altering probe in SDSL analysis, has the advantage of being much smaller than 10-DN. Fortunately, the controls described above show that the amount of 10-DN used does not greatly perturb bilayers. The need to incorporate 10-DN into bilayers prior to bilayer formation is another difference between DQA and SDSL analysis. It necessitates the preparation of separate samples containing 10-DN, which in principle increases experimental error to some degree.

It is also important not to overlook two important advantages of DQA relative to SDSL analysis. The first is the above-noted relative ease with which multiple populations at different depths may be detected. The second is the ability of DQA to measure depth with widely available fluorescence instrumentation. Overall, DQA should be of particular use for application to membrane-interacting polypeptides.

REFERENCES

1. Meijer, A. B., Spruijt, R. B., Wolfs, C. J., and Hemminga, M. A. (2001) *Biochemistry 40*, 5081−5086.
2. Opella, S. J., Ma, C., and Marassi, F. M. (2001) *Methods Enzymol. 339*, 285−313.
3. Ding, F. X., Schreiber, D., VerBerkmoes, N. C., Becker, J. M., and Naider, F. (2002) *J. Biol. Chem. 277*, 14483−14492.
4. Kachel, K., Ren, J., Collier, R. J., and London, E. (1998) *J. Biol. Chem. 273*, 22950−22956.
5. Ren, J., Lew, S., Wang, Z., and London, E. (1997) *Biochemistry 36*, 10213−10220.
6. Chattopadhyay, A., and London, E. (1987) *Biochemistry 26*, 39−45.
7. Abrams, F. S., and London, E. (1993) *Biochemistry 32*, 10826−10831.
8. London, E., and Ladokhin, A. S. (2002) in *Current Topics in Membranes* (Benos, D., and Simon, S., Eds.) Vol. 52, pp 89−115, Elsevier, San Diego.
9. Altenbach, C., Marti, T., Khorana, H. G., and Hubbell, W. L. (1990) *Science 248*, 1088−1092.
10. Bolen, E. J., and Holloway, P. W. (1990) *Biochemistry 29*, 9638−9643.
11. Eftink, M. R., and Ghiron, C. A. (1976) *Biochemistry 15*, 672−680.
12. Kachel, K., Asuncion-Punzalan, E., and London, E. (1995) *Biochemistry 34*, 15475−15479.
13. Lew, S., and London, E. (1997) *Anal. Biochem. 251*, 113−116.
14. Lakowicz, J. (1999) *Principles of Fluorescence Spectroscopy*, 2nd ed., Plenum, New York.
15. Johannsson, A., Keightley, C. A., Smith, G. A., Richards, C. D., Hesketh, T. R., and Metcalfe, J. C. (1981) *J. Biol. Chem. 256*, 1643−1650.
16. Lewis, B. A., and Engelman, D. M. (1983) *J. Mol. Biol. 166*, 211−217.
17. Chattopadhyay, A., and London, E. (1988) *Biochim. Biophys. Acta 938*, 24−34.
18. Blok, M. C., Van Deenen, L. L., and De Gier, J. (1977) *Biochim. Biophys. Acta 464*, 509−518.

19. London, E., and Feigenson, G. W. (1981) *Biochemistry 20*, 1932−1938.
20. Bangham, A. D., De Gier, J., and Greville, G. D. (1967) *Chem. Phys. Lipids*, 225−246.
21. Homan, R., and Eisenberg, M. (1985) *Biochim. Biophys. Acta 812*, 485−492.
22. Haydon, D. A., Hendry, B. M., Levinson, S. R., and Requena, J. (1977) *Nature 268*, 356−358.
23. Ren, J., Lew, S., Wang, J., and London, E. (1999) *Biochemistry 38*, 5905−5912.
24. Killian, J. A. (1998) *Biochim. Biophys. Acta 1376*, 401−415.
25. Webb, R. J., East, J. M., Sharma, R. P., and Lee, A. G. (1998) *Biochemistry 37*, 673−679.
26. Jiang, J. X., and London, E. (1990) *J. Biol. Chem. 265*, 8636−8641.
27. Dhanvantari, S., Arnaoutova, I., Snell, C. R., Steinbach, P. J., Hammond, K., Caputo, G. A., London, E., and Loh, Y. P. (2002) *Biochemistry 41*, 52−60.
28. Caputo, G. A., and London, E. (2003) *Biochemistry 42*, 3275−3285.
29. Wimley, W. C., and White, S. H. (1992) *Biochemistry 31*, 12813−12818.
30. Kaiser, R. D., and London, E. (1998) *Biochemistry 37*, 8180−8190.
31. Shatursky, O., Heuck, A. P., Shepard, L. A., Rossjohn, J., Parker, M. W., Johnson, A. E., and Tweten, R. K. (1999) *Cell 99*, 293−299.
32. Ladokhin, A. S. (1999) *Biophys. J. 76*, 946−955.
33. Blatt, E., and Sawyer, W. H. (1985) *Biochim. Biophys. Acta 822*, 43−62.
34. London, E. (1982) *Mol. Cell. Biochem. 45*, 181−188.
35. Ladokhin, A. S. (1997) *Methods Enzymol. 278*, 462−473.
36. Moro, F., Goni, F., and Urbaneja, M. A. (1993) *FEBS Lett. 330*, 129−132.

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