Groups with Polar Characteristics Can Locate at Both Shallow and Deep Locations in Membranes: The Behavior of Dansyl and Related Probes[†]

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ABSTRACT: To understand the relationship between the chemical structure of polar molecules and their membrane location, the behavior of dansyl (dimethylaminonaphthalenesulfonyl) and related polar fluorescent probes was examined. The depth of these probes in lipid bilayers was determined by parallax analysis of fluorescence quenching [Chattopadhyay and London (1987) *Biochemistry 26*, 39−45; Abrams & London, *Biochemistry* (1993) *32*, 10826−10831]. Quenching was measured for dansyl groups: (1) attached to the polar headgroup of PE, (2) linked to an alkyl chain, (3) attached to the end of a fatty acyl chain, and (4) attached to the polar headgroup of PE via a spacer group. In all cases, the dansyl probes located in the polar headgroup region, 19−21 Å from the bilayer center. This shows the dansyl group has a strong tendency to seek a shallow location in the polar headgroup region. The only exception to this pattern was in the case of a dialkylated dansyl, for which two populations were observed. One population was at the polar headgroup level, but the second was deeply buried in the acyl chain region. To see if the polar sulfonamide group of dansyl influences depth, a structurally related probe substituting a thiocarbamoyl linkage, dimethylaminonaphthalenethiocarbamoyl (dantyl)-labeled PE, was synthesized. Dantyl groups were located deeper than dansyl groups, 13−16 Å from the bilayer center. There was an even more dramatic difference in depth between dansyl and mansyl (methylanilinonaphthalenesulfonyl) derivatives. Mansyl probes, which have an extra phenyl group relative to dansyl, were found to locate deeply within the acyl chain region of the bilayer (6−7 Å from the bilayer center) when attached to the polar headgroup of PE. Thus, the membrane location of polar groups depends strongly on the details of their chemical structure, and it is possible for a polar group to locate both at shallow and deep locations. These results suggest the energy to bury a polar moiety in the hydrophobic part of the bilayer is not prohibitively high. This contrasts to the behavior of charged groups, which appear to be restricted to shallow locations in membranes. In this report, the effect of populations at two different depths on the parallax analysis is also considered.

To understand membrane structure, the rules governing the location of molecules within the bilayer must be determined. Both proteins and lipids take on structures and interactions with other molecules that are influenced by their location within the bilayer. Knowledge of membrane location is especially important for fluorescent probes designed to explore membrane properties at specific depths within a membrane. Most studies of the location of fluorescent probes in membranes rely on indirect measures, such as shifts in emission maxima to estimate depth (*1*). This method cannot precisely locate a fluorophore because (1) emission λ_{max} can reflect specific interactions between the fluorophore and surrounding molecules as well as polarity (*2*, *3*); and (2) no isotropic solvent can exactly mimic bilayer behavior (*4*). These factors can result in anomalous wavelength shifts (*3*, *4*).

Our group developed parallax analysis of fluorescence quenching as a method to allow more precise determination of the transverse location of fluorophores in membranes (*5*−*7*). This method involves fluorescence quenching measurements using phospholipids carrying a nitroxide (spin) label at different locations in the bilayer. The ratio of the fluorescence intensity in the presence of a shallow and a deep quencher is used to calculate fluorophore depth. This method has been used to determine the membrane location of NBD attached to the polar headgroup or the acyl chain of phospholipids (*5*, *7*), anthroyloxy, and carbazole groups attached to fatty acids (*6*−*8*), tryptophan and tyrosine analogues (*9*), and anthracene probes linked to various polar groups (*10*). Several groups have also used the method to examine the membrane location of polypeptides and membrane-inserted proteins (*3*, *11*−*19*).

In this paper, the location of the polar dansyl group and structurally related probes was studied. This family was chosen both because they are commonly used membrane probes, and to allow examination of the behavior of strongly polar groups in membranes. The structure of both the probes and the molecule to which they are linked was varied. It was found that both of these variables can affect probe depth. From these studies and previous work, general rules that

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govern the depth of fluorescent probes in membranes are proposed. This should allow improved prediction of the location of molecules in membranes and the development of strategies for modifying chemical structure to localize molecules at specific depths in a membrane.

EXPERIMENTAL PROCEDURES

Materials. The triethylammonium salts of dansyl-DHPE[1] and dansyl-X-DHPE were purchased from Molecular Probes. Their purity was checked by TLC on silica gel plates using chloroform:methanol:water (65:25:4 v/v) as the developing solvent. Each gave a single colored spot at a level of loading on the plate that indicated less than 1% impurity. Their identity was confirmed by their emission and excitation spectra (see below), which corresponded to expected values. Stock solutions were prepared in ethanol, and concentration was confirmed by measuring the absorbance of the fluorophore using the reported molar extinction coefficient (M^{-1} cm^{-1}) at the appropriate wavelengths (*20*): for dansyl-DHPE at 333 nm, 4500; dansyl-X-DHPE at 346 nm, 3600. Hexadecanoyldansyl, dihexadecanoyldansyl, and dansylundecanoic acid were also purchased from Molecular Probes. The purity of these molecules was checked by TLC on silica gel plates using $CHCl_3$ as the developing solvent. All gave one colored spot.

Nitroxide-labeled PCs and DOPC were purchased from Avanti Polar Lipids. The concentration of phospholipids was determined either by phosphate assay after digestion (*21*, *22*) or by dry weight. The nitroxide concentration was assayed by fluorescence quenching of 2-AS and 12-AS. The percentage of uncalibrated nitroxide-labeled lipid that had to be incorporated into multilamellar vesicles to give the same quenching as an ESR-calibrated sample with 15% nitroxide-labeled lipid (*7*) was determined. The ratio of nitroxide groups to lipid was generally found to be in the range 0.8−0.9.

Synthesis of Other Fluorescent-Labeled Phospholipids. 4-Dimethylaminonaphthyl-1-isothiocyanate and 6-(*N*-methylanilino)naphthalene-2-sulfonyl chloride were purchased from Molecular Probes. DOPE, DHPE, and DOPD were purchased from Avanti Polar Lipids. An excess (up to 65%) of the fluorophore was reacted with PE following the procedure of Fung and Stryer (*23*) with slight modifications. The mixture of fluorophore and lipid was dissolved in 1 mL of $CHCl_3$, and 100 μL of triethylamine (Fisher Scientific) was added. The reaction mixture was then vortexed at low speed for 1 h at room temperature in the dark. The product was purified and isolated by TLC on a 20 × 20 cm preparative silica gel 60 plate (E. Merck) previously activated by heating to 140 °C for 1 h. The plate was developed in chloroform:methanol:water (65:25:4 v/v). The main band was scraped off the plate and extracted first with chloroform:methanol (2:1 v/v) and then with ethanol. The extracts were collected and dried under nitrogen. Ethanol was added to the dried samples and the solutions were filtered to remove the silica gel. The phospholipid concentration was determined by phosphate assay as above.

Preparation of Samples for Depth Measurements. SUV prepared by octylglucoside dilution were used for depth measurements. First, a fluorophore/octylglucoside stock solution was prepared by mixing the desired amount of fluorophore and octylglucoside in ethanol. The mixture was dried under N_2, and the dried mixture was hydrated with 10 mM sodium acetate and 150 mM NaCl, pH 6.6−7, for the phospholipids; 10 mM sodium acetate and 150 mM NaCl, pH 4.6−5 or 10 mM glycine, 150 mM NaCl, pH 10, for dansylundecanoic acid; and 10 mM sodium phosphate and 150 mM NaCl, pH 7, for the other fluorophores, unless otherwise noted. The volume of buffer added at this stage gave an octylglucoside concentration of 50 mM and a fluorophore concentration 50 times that in the final l mL samples (see below).

Next, aliquots of DOPC and nitroxide-labeled PC dissolved in ethanol were mixed with 1 μmol of octylglucoside. Four sets of SUV samples were prepared: one containing DOPC and the other three containing 15 mol % of the appropriate nitroxide-labeled PCs mixed with DOPC.[2] The total amount of lipid was 100 nmol except for experiments with dansyl and dantyl, where 200 nmol of lipid was used. The lipid/octylglucoside mix was dried under N_2. Then, 20 μL of the fluorophore/octylglucoside stock solution was added and mixed until the lipids dissolved.

The samples were brought to 1 mL by adding 980 μL of the desired buffer (the same one used to dissolve the fluorophore) and vortexed. These samples were prepared in duplicate. The final concentration of the fluorescent-labeled phospholipids was 0.5 μM for the mansyl-labeled phospholipids, 1 μM for dansyl-DHPE and dansyl-X-DHPE, and 2 μM for the dantyl-labeled phospholipids and the other dansyl probes. The final octylglucoside concentration was 2 mM. The final total lipid concentration of these solutions was 100 μM for the mansyl derivatives and 200 μM for the dansyl and dantyl-labeled molecules. Under these conditions essentially all fluorescence arises from model membrane-bound molecules (not shown). Single background samples without the fluorophore were prepared similarly except 20 μL of buffer was added instead of the fluorophore/octylglucoside stock solution.

Fluorophore depths derived from vesicles made by the octylglucoside procedure were very similar to that observed when vesicles were prepared by the ethanol dilution procedure (not shown) (*9*).

Fluorescence Measurements. Samples were placed in a 1 cm path-length quartz cuvette, and their fluorescence intensity was measured with a Spex 212 Fluorolog spectrofluorimeter operating in ratio mode. The excitation and

[1] Abbreviations: 2- or 12-AS, 2- or 12-(9-anthroyloxy)stearic acid; dansyl-, *N*-(5-dimethylaminonaphthalene-1-sulfonyl); dansyl-X-, *N*-(6-(5-dimethylaminonaphthalene-1-sulfonyl)amino)hexanoyl; dantyl-, *N*-(4-dimethylaminonaphthalene)-1-thiocarbamoyl); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DHPE, 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; DOPD, 1,2-dioleoyl-*sn*-glycero-3-(N-[12-aminododecanoyl]-phosphoethanolamine); ESR, electron spin resonance; mansyl, *N*-(6-(*N*-methylanilino)naphthalene-2-sulfonyl); NBD, 7-nitro-2,1,3-benzoxadiazol-4-yl; PE, phosphatidylethanolamine; 5- or 12-SLPC, 1-palmitoyl-2-(5- or 12-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; SUV, small unilamellar vesicles; Tempocholine, 4-(*N*,*N*-dimethyl-*N*-(2-hydroxyethyl))ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl; TempoPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphotempocholine; TLC, thin-layer chromatography.

[2] The nitroxide-labeled lipid contains some molecules that lack a nitroxide group. The amount of nitroxide-labeled lipid is adjusted to give 15% nitroxide-containing molecules and 85% other lipid molecules (DOPC plus inactive "quencher").

emission slits were set between 1.25 and 5.0 mm (2.25−9 nm band-pass) so as to prevent saturation of the photomultiplier tube. Fluorescence was measured at the uncorrected λ_{max} for excitation and emission. The following excitation wavelengths were used: 328 nm for the mansyl-labeled lipids, 333 nm for dansyl-DHPE, 337 nm for hexadecyldansyl, 338 nm for dantyl-DOPE, 339 nm for dansylundecanoic acid, 340 nm for dantyl-DOPD, dihexadecyldansyl, and dansyl-X-DHPE, and 344 nm for dantyl-DHPE. Emission was monitored at the following wavelengths: 433 nm for mansyl-DOPE and mansyl-DOPD, 435 nm for mansyl-DHPE, 451 nm for dantyl-DOPE, 454 nm for dantyl-DHPE, 455 nm for dantyl-DOPD, 510 nm for dansyl-DHPE, 513 nm for dansyl-X-DHPE, 518 nm for dihexadecyldansyl, and 520 nm for hexadecyldansyl and dansylundecanoic acid. Fluorescence was measured at room temperature and averaged over three 6−10 s readings. The fluorescence intensity from duplicate SUV samples containing the fluorophore was averaged, and the intensity of the background samples without fluorophore was subtracted. (Background intensity was less than 3% of the sample intensity in all cases).

The pK_a of the carboxyl group of membrane bound dansylundecanoic acid was determined by measuring fluorescence intensity as a function of pH (*8*). A pK_a value of close to 7 was obtained.

The fluorescence intensities in the presence of the quenchers were substituted into the parallax equation: $z_{cf} = L_{c1} + [((-1/\pi C)\ln(F_1/F_2)) - L_{21}^2]/2L_{21}$ (*5*, *7*) to calculate the depth of the fluorescent group. In this equation, z_{cf} is the distance of the fluorescent group from the center of the bilayer, L_{c1} is the distance of the shallow quencher from the bilayer center, C is the concentration of the quencher in molecules per unit area, F_1 and F_2 are the fluorescence intensities in the presence of the shallow and deep quencher, respectively (or equivalently these intensities divided by that in the absence of quencher F_1/F_o, F_2/F_o), and L_{21} is the difference in depths of the shallow and deep quencher.

In general, the quenching by the pair of quenchers that quenches the most (i.e., the TempoPC/5 SLPC pair or 5 SLPC/12SLPC pair) was used to calculate z_{cf} (*7*). When the difference in TempoPC and 12SLPC quenching was 5−6% of F/F_o values, i.e., $0.95 < F_{TempoPC}/F_{12SLPC} < 1.05$, then the average of the z_{cf} obtained with the TempoPC/5SLPC pair and 5SLPC/12SLPC pair was used. The distances of the quenchers from the bilayer center used were 5.85 Å for 12SLPC, 12.15 Å for 5SLPC, and 19.5 Å for TempoPC (*6*, *7*).

Control Experiments To Determine the Chemical Stability of the Fluorescent Lipids. Fluorophore chemical stability was checked by comparing fluorescence intensity in vesicle samples prepared with or without 15% nitroxide-labeled lipids after dissolving 100 μL of each vesicle sample with 900 μL ethanol to dissolve the vesicles and eliminate quenching. Samples were vortexed just prior to measurement of fluorescence intensities. In all cases, after dissolving vesicle samples in ethanol a ratio close to 1 was found for fluorescence in the presence of each quencher to that in its absence. This indicates that the fluorophores used in this study did not react with the nitroxides under the conditions used in this report.

Cobalt Quenching Experiments. The quenching of dansyl probes by $CoCl_2$ was measured by preparing ethanol dilution vesicles (*9*) containing 2 μM probe and 200 μM lipid in Co^{2+}-containing solutions. Samples were prepared by dilution of lipid dissolved in ethanol with 10 mM sodium acetate and 150 mM NaCl, pH 4.1 containing 0−300 mM $CoCl_2$. Prepared in this way, $CoCl_2$ was present both in the vesicle lumen and the external solution. (Co^{2+} was found to remain soluble at low pH.) Fluorescence was measured with an excitation wavelength of 337 nm and emission at 450 or 570 nm.

Table 1: Fluorescence Quenching Values and Calculated Location of Fluorescent-Labeled Probes in Membrane Bilayers[a]

fluorescent probe	F_{TC}/F_o	F_5/F_o	F_{12}/F_o	z_{cf} (Å)
dansyl-DHPE	0.35	0.48	0.52	19
dansyl-X-DHPE	0.37	0.50	0.53	19
dansyl undecanoic acid, pH 4.6 (protonated)	0.31	0.48	0.48	20.5
dansyl undecanoic acid, pH 10 (ionized)	0.37	0.48	0.51	18.5
hexadecyldansyl	0.30	0.47	0.48	20
dihexadecyldansyl	0.36	0.51	0.46	(19.5)[b]
dantyl-DOPE	0.43	0.43	0.49	16
dantyl-DHPE	0.38	0.39	0.48	16
dantyl-DOPD[c]	0.42	0.42	0.44	13
mansyl-DOPE	0.51	0.50	0.40	6.5
mansyl-DHPE	0.51	0.50	0.41	7
mansyl-DOPD	0.57	0.58	0.47	6.5

[a] The quenching values shown are the average of at least two independent experiments, each with duplicate samples. The standard deviation in z_{cf} was at most ±0.5 Å. Unless otherwise noted, the pair of spin-label quenchers which quenches most is used to calculate fluorophore distance from the bilayer center (z_{cf}) using the equation in Abrams and London (*7*). F_{TC}/F_o, F_5/F_o, and F_{12}/F_o are F/F_o values giving the ratio of fluorescence intensity in vesicles containing 15 mol % TempoPC, 5SLPC, or 12SLPC, respectively, to that in vesicles lacking nitroxide. [b] This is the depth of the primary shallow population. The deep population has a depth of 6−7 Å from the bilayer center (see Figure 3). [c] In this case, the degree of quenching of TempoPC, 5-SLPC, and 12-SLPC were indistinguishable within experimental error (less than 5−6% difference in fluorescence intensity). In such cases the average of the depths obtained with TempoPC/5-SLPC and 5-SLPC/12-SLPC was used to calculate z_{cf} (see ref 7).

RESULTS

Measuring the Depth of Fluorescent Lipids in Model Membranes. The chemical structures of the fluorescent probes studied are shown in Figure 1. Table 1 gives the fluorescence quenching values for these lipids in unilamellar vesicles containing 15% of shallow (TempoPC), medium (5SLPC), or deep (12SLPC) nitroxide-labeled lipids. Strong fluorescence quenching was observed for all fluorescent molecules. The amount of fluorescence quenching obtained with the different nitroxide-labeled lipids was then used to calculate the distance of the fluorophores from the center of the bilayer (z_{cf}) using the parallax equation (see Experimental Procedures).

Depth of Dansyl Membrane Probes. Table 1 shows that a dansyl attached to the headgroup of PE (dansyl-DHPE) located shallowly within the polar headgroup region of the bilayer. Quenching was strongest by the shallow TempoPC, and a z_{cf} value of 19 Å was obtained. This is about the same depth we have observed for charged fluorescent probes attached to the PE amino group (unpublished observations) and for NBD, a strongly polar uncharged probe, attached to the PE amino group (*7*). To see if the dansyl group occupied

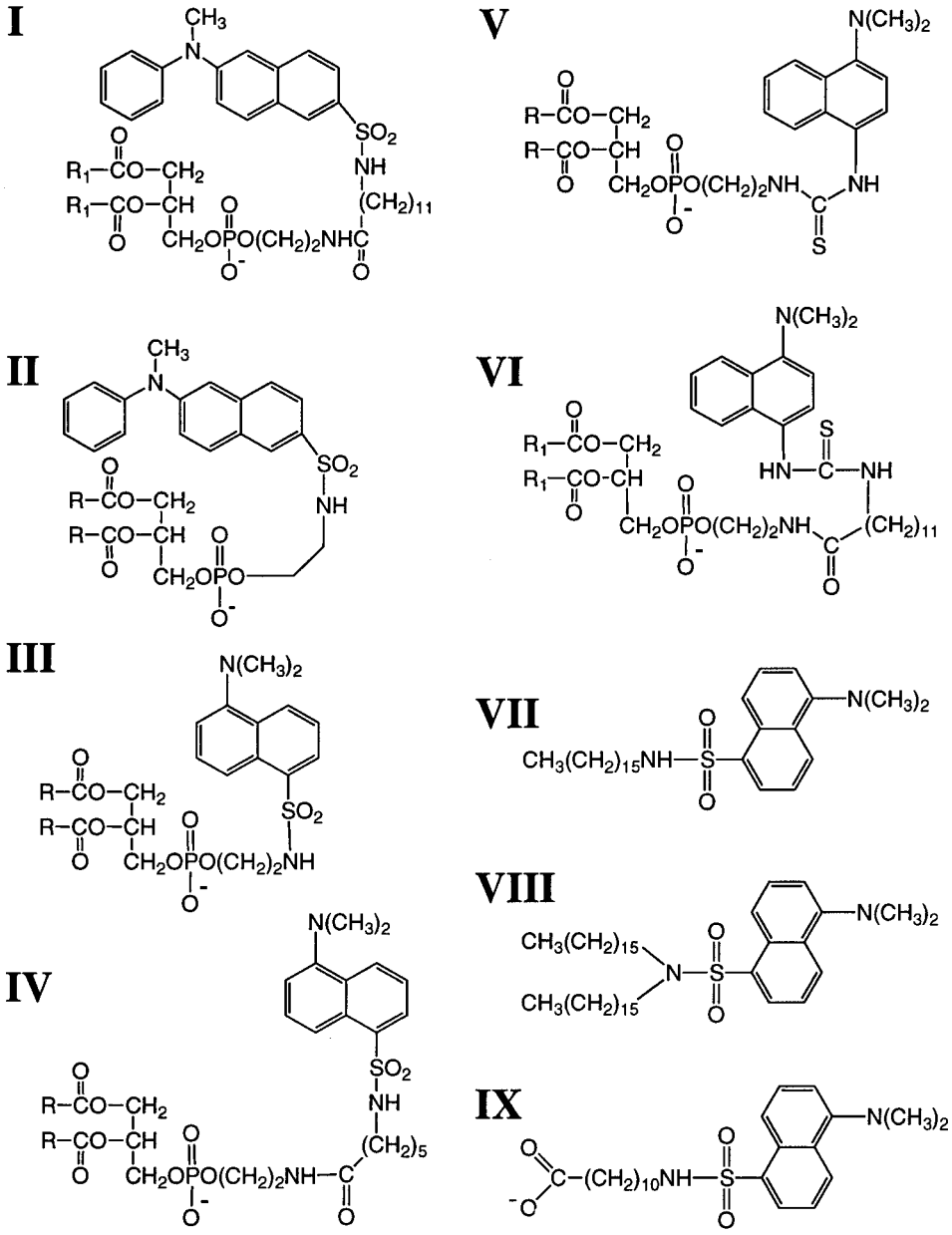

FIGURE 1: Chemical structures of the fluorescent probes used in this study. I, mansyl-DOPD; II, mansyl-DHPE; III, dansyl-DHPE; IV, dansyl-X-DHPE; V, dantyl-DHPE; VI, dantyl-DOPD; VII, hexadecyldansyl; VIII, dihexadecyldansyl; IX, dansylundecanoic acid. RCOO = hexadecanoyl; R_1COO = oleoyl. Mansyl-DOPE and dantyl-DOPE are not illustrated.

a shallow location simply due to its attachment to the polar headgroup, the depth of a dansyl attached to the end of the acyl chain of a fatty acid (dansyl undecanoic acid) was measured. A shallow dansyl location was observed regardless of whether the fatty acid carboxyl was protonated or ionized. This shows that dansyl, like NBD (*5*), is sufficiently polar to loop up toward the membrane surface. A shallow dansyl depth was also found for hexadecyldansyl, in which the dansyl is attached directly to an alkyl chain, and for dansyl-X-PE, in which dansyl is attached to a linker with the potential to fold back into the nonpolar region or extend further out into the aqueous phase. Together these results show dansyl probes have a strong intrinsic tendency to locate shallowly in the polar headgroup region rather than deeper or in the aqueous phase around the bilayer.

A partial exception to this behavior was seen for the dihexadecyldansyl probe. This dansyl exhibited stronger quenching by the shallow quencher TempoPC and the deep quencher 12SLPC than that for the intermediate depth quencher 5SLPC. This pattern is seen when there are two probe populations at two depths, one shallow and one deep (*24*). The presence of two dansyl populations at different depths was confirmed by subsequent experiments (see below). Presumably, the attachment of a second alkyl chain renders the dansyl group sufficiently hydrophobic so that its energy at the surface or deeply buried is similar.

The Lipid-Linking Group Affects Dansyl Depth: Behavior of Dantyl Labeled Lipids. The behavior of dihexadecyldansyl suggests that the depth preference of a strongly polar probe can be modified. We next attempted to identify structural features of the dansyl group that influence its localization. The dansyl derivatives examined above all were linked to the rest of the molecule via a sulfonamide linkage. This group is polar and can form hydrogen bonds, which

might have a strong influence on depth. To see if this was the case, the effect of attaching dimethylaminonaphthalene portion of the dansyl probe to PE via a thiocarbamide linkage (i.e., a dantyl probe) was examined. The thiocarbamide group has two fewer oxygens than the sulfonamide group and might be expected to result in a less polar molecule with a lesser tendency to hydrogen bond.

Consistent with a lesser polarity, the thiocarbamide-linked dantyl group attached to PE appeared to locate 3 Å more deeply than PE-linked dansyl (Table 1). The dantyl group located a few angstroms more deeply in dantyl-DOPD, in which there is a 12-carbon linker between the dantyl group and the PE than in dantyl-DOPE.

Dansyl vs Mansyl: Fluorophores with Similar Structures Can Locate at Very Different Depths. To determine if the location of the polar dansyl fluorophore in membranes is sensitive to changes in fluorophore structure, the depth of PE-linked dansyl and mansyl groups was compared. Dansyl and mansyl are closely related except that, in the latter, one of the methyl groups linked to nitrogen is replaced by a phenyl ring, making it more hydrophobic, and the sulfonamide and nitrogen positions on the naphthalene core are shifted (Figure 1). Table 1 shows this has a marked effect on depth, with mansyl attached to PE being located 6−7 Å from the bilayer center while the analogous dansyl-PE is at 19 Å from the bilayer center. A deep location was also observed for mansyl-DOPD, in which the mansyl group is linked to the PE through the 12-carbon spacer. This suggests that the location of the mansyl probe reflects an intrinsic tendency to be buried deeply within the acyl chain region of the bilayer.

The Effect of Other Factors on Probe Depth. Acyl chain structure has the potential to affect the depth of fluorophore attached to the polar headgroup. To see if this was the case, depth of dantyl and mansyl covalently linked to the headgroup of DOPE and DHPE was compared. These lipids differ in acyl chain structure, which includes double bonds in the case of DOPE. Table 1 shows that the depth of both of these probes is basically unaffected by whether oleoyl (in DOPE) or palmitoyl (in DHPE) acyl chains are present. The effect of lipid composition on the depth of dansyl-DHPE was also examined. The depth of dansyl-DHPE in membranes composed of 50 mol % PG and 50 mol % PC was similar to that obtained with 100 mol % PC (not shown). Finally, the absence of NaCl also had no effect on dansyl-DHPE depth, showing that ionic strength had little influence on depth (not shown).

Dependence of Apparent Depth on Excitation and Emission Wavelength. Previous studies have shown that the apparent membrane depth can be dependent on the wavelengths used to measure fluorescence (*7*, *8*, *10*). Generally, fluorophores appear, at most, slightly closer to the membrane surface when depth is measured at longer wavelengths. We also investigated the depth dependence on excitation and emission wavelength for several of the fluorescent-labeled lipids used in this study. A slightly shallower depth at the long wavelength end of emission spectra was found for most dansyl and mansyl-labeled probes (not shown and Figure 2A).

Very different behavior was observed for dihexadecyldansyl (Figure 2B). It demonstrated a strong wavelength dependence of depth, as shown qualitatively by the relative

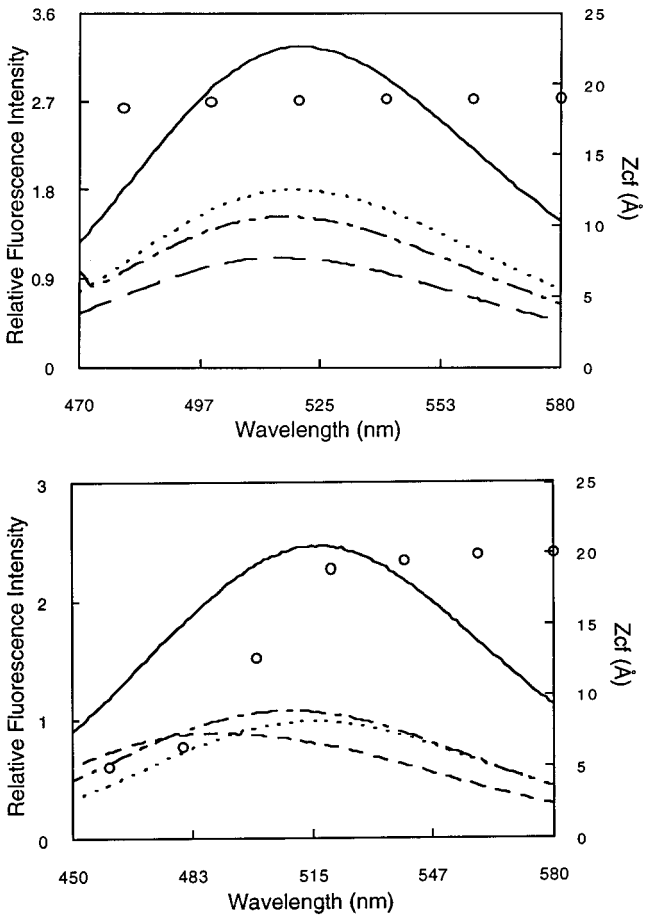

FIGURE 2: Dependence of apparent depth on wavelength for hexadecyldansyl and dihexadecyldansyl. Emission spectra (lines) and z_{cf} values (○) are shown for (A, top) hexadecyldansyl and (B, bottom) dihexadecyldansyl. Spectra are shown for probes incorporated into (—) DOPC vesicles or vesicles containing (- - -) 15 mol % TempoPC, (—•—•—) 15 mol % 5SLPC or (•••) 15 mol % 12SLPC.

strength of nitroxide quenching. At long wavelength, the shallow TempoPC quenched most strongly, whereas at short wavelengths, the deep 12SLPC quenched most strongly. This wavelength dependence was not seen for any other probe, including hexadecyldansyl.

Dihexadecyldansyl Behavior Can be Explained by Different Population with Different Emission Maxima. The behavior of dihexadecyldansyl can be explained by there being two major populations of dansyl molecules at different depths, as noted above. To explain the wavelength dependence, the population of dansyl molecules that is deep would have to give rise to preferentially blue-shifted fluorescence (i.e., emission at shorter wavelengths), and the population that is shallow would have to give rise to more red-shifted fluorescence. This behavior is exactly what would be expected given the fact that the more deeply buried population should be in a nonpolar environment and so should be expected to give blue-shifted emission relative to the shallower population which would be in a polar environment.

Experiments with samples prepared in the presence of the aqueous quencher Co^{2+} (Figure 3) were consistent with this model. The amount of exposure to Co^{2+} can measure how close a fluorescent probe is to the membrane surface (*25*). Quenching at 570 nm, where the shallow dihexadecyldansyl molecules dominate fluorescence, was equal for hexadecyl-

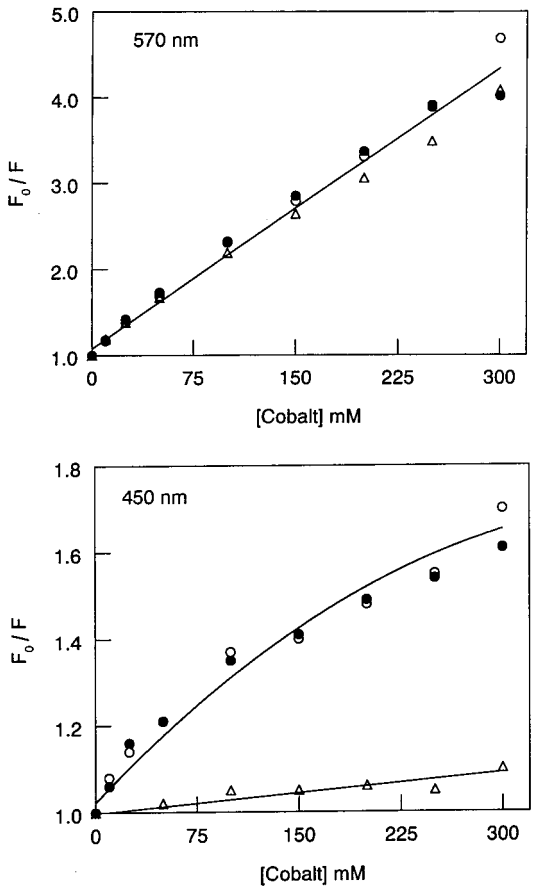

FIGURE 3: Cobalt quenching of dansyl probes inserted into model membrane vesicles. Vesicles were composed of DOPC. (A, top) Emission at 570 nm. (B, bottom). Emission at 450 nm. (▲) Dihexadecyldansyl; (○) dansylundecanoic acid; (●) hexadecyldansyl. F_o/F is the ratio of fluorescence in the absence of $CoCl_2$ to that in its presence. The vesicles are prepared such that $CoCl_2$ is present both in the internal lumen and external solution (see Experimental Procedures).

dansyl and dihexadecyldansyl, consistent with their equally shallow apparent depths at this wavelength. However, at 450 nm, where the deep population dominates for dihexadecyldansyl, there was much weaker Co^{2+} quenching for dihexadecyldansyl than for hexadecyldansyl.[3] Furthermore, dansylundecanoic acid, which appears to locate about as shallowly as hexadecyldansyl (Table 1), exhibited Co^{2+} quenching identical to that of hexadecyldansyl (Figure 3).

DISCUSSION

The Depth of Molecules in Membranes: Classification of Polar Molecules. This report indicates that the depth of polar groups in a membrane reflects a combination of the probe properties and the nature of their attachment to the remainder of the molecule. This distinguishes them from charged probes, which locate at the polar surface under all conditions explored to date (unpublished observations). Combining the results of this study with previous work allows us to divide polar molecules into two classes with different behaviors. *Class I, strongly polar:* this class of molecules includes NBD and dansyl. These probes locate at very shallow depths within the polar headgroup region. In fact, they tend to locate close at the same depth as charged probes. Both dansyl and NBD (*2*, *7*, *25*, *28*) have a strong tendency to loop up into the polar region of the membrane even if attached to the end of fatty acyl chains. However, in some cases their tendency to locate at the polar surface can be overcome by how they are attached to the remainder of the molecule (*5* and Results). *Class II, weak to moderate polarity*: this class includes molecules such as carbazole, phenol, indole, anthroyloxy, and dantyl probes. By themselves, such molecules locate close to the polar/hydrocarbon boundary, i.e., more deeply than the molecules in class I (*1*, *9*, *29*, *30* and Results). Their attachment to acyl chains generally overcomes their surface seeking properties, allowing their burial deep within the hydrocarbon region of the bilayer (*6*−*9*).

The mansyl group falls into neither of these classes. It probably orients such that its nonpolar portion is deeply buried in the membrane. Why is the predominant depth of mansyl so much deeper than dansyl given the overall similarity of their structures? One factor is that mansyl is more hydrophobic than dansyl due to the extra phenyl group. Another is that mansyl can orient with its polar sulfonamide portion close to the surface and, at the same time, have its aromatic rings inserted deeply in the bilayer.

Dihexadecyldansyl behavior demonstrates that some fluorescent molecules can exist in populations at two distinct depths. This behavior indicates that for dihexadecyldansyl, shallow and deep dansyl locations have nearly equal energy. Why this should be is unclear. One possibility is that when the dansyl is shallow, it interacts most favorably with its polar environment, and its deep location allows favorable packing interactions between the dihexadecyl chains and the surrounding lipids.

The unexpected behavior of the mansyl and dihexadecyldansyl probes show that it can be dangerous to predict probe behavior in the absence of experimental information. In some cases, small changes in probe structure, or how it is linked to the rest of a molecule, can result in large changes in its membrane location.

Comparison to Previous Studies of Fluorophore Location. There is strong evidence that the parallax analysis gives accurate depths from a number of studies. There is good agreement of the depths obtained by the parallax analysis for anthroyloxy fatty acids, NBD, Trp and Tyr analogues, and anthracene derivatives with information obtained, often much more indirectly and/or at a lower level of resolution, by other methods (*5*, *10*). Direct calibration with a series of Trp-containing transmembrane peptides has also been recently demonstrated (*19*).

Comparison of the results for dansyls in this study and other information concerning their location in bilayers are also in reasonable agreement. The depth of dansyl groups has been roughly estimated from a comparison of their

[3] Notice there is more quenching overall at 570 than at 450 nm. This is due to the fact that dansyl molecules fluorescing at short wavelengths have a shorter excited-state lifetime than those fluorescing at long wavelengths (*26*, *27*). Collisional quenching is proportional to *both* the number of collisions per unit time (and thus how close fluorophore and quencher can approach) and lifetime (*2*). Using a Spex τ2 instrument for all three dansyl derivatives used in the Co^{2+} quenching experiments we measured a lifetime of 16−17 ns at 570 nm, and a broad lifetime distribution with an average value of approximately 3−5 ns at 450 nm.

λ_{max} values in model membranes relative to that in solvent of varying polarity. Such studies have indicated that dansyl and related derivatives are located somewhere in the polar headgroup region (*1*, *31*) in agreement with our results.

Fluorescence Quenching and Populations of Molecules at Different Depths in the Membrane. There are several limitations on our ability to exactly interpret the fluorophore behavior from experiments in which two populations at different depths are present. First, if two populations are present z_{cf} should reflect the average depth from which fluorescence originates, rather than the average depth of the fluorescent molecules themselves. This in turn depends on the quantum yield and wavelength dependence of fluorescent intensity for each species. Therefore, quantifying the exact amount of each species present is difficult. In addition, we cannot state whether the multiple populations of fluorophore occur in the ground state or just in the excited state. On the other hand, in terms of using fluorophores as membrane probes, it is the depth from which the fluorescence being measured originates that is important, and this is the parameter that can be determined from quenching experiments.

It should also be noted that it is difficult to specify exact distributions from the fluorescence quenching data. For example, we cannot rule out a third population of dihexadecyldansyl molecules with an intermediate dansyl depth. In addition, for the other probes studied, we cannot rule out minor subpopulations at depths far from the value of z_{cf}. There is a hint that such subpopulations may exist from the quenching data. For a single population of fluorophores, the quenching by the quencher farthest from the fluorophore should be significantly less than that for the quencher at the intermediate distance from the fluorophore, as we have observed previously (*6*−*10*). In contrast, for dansyl and related fluorophores, we observe that the quenching by the two quenchers farthest from the fluorophore depth is often very similar, as could be caused by a subpopulation at a depth far from that of the main population. Further evidence that there can be minor subpopulations come from the Co^{2+}-quenching profiles for hexadecyldansyl and dansylundecanoic acid. Even though they are predominantly shallow, the curvature of their quenching profiles at 450 nm indicates the presence of at least two populations.

One question that arises from these possibilities is how the presence of different subpopulations affects the depth measured by the parallax analysis. Specifically, is measured z_{cf} equal to the true average z_{cf} (weighted by fluorescence intensity of the relevant species) when there are two populations at distinct average depths? As shown in the Appendix, unless the deep and shallow populations are almost equal in population *and* are very far away from each other, depth calculations with the appropriate quencher pair will give a measured z_{cf} close to the average z_{cf}. The few cases in which there are nearly equal amounts of deep and shallow fluorophore populations can be identified by their having almost equal quenching by TempoPC and 12 SLPC (a ratio of F/F_o values within 5% of each other). In these cases, the average of the depth obtained with the TempoPC/5SLPC pair and the 5SLPC/12SLPC pair can be used to obtain a more accurate average depth (*7*, *10*).

APPENDIX: EFFECT OF TWO POPULATIONS OF A FLUOROPHORE AT DIFFERENT DEPTHS ON DEPTH MEASURED REPORTED BY THE PARALLAX ANALYSIS

We previously found (*6*) that where there is an even distribution of fluorophores over a range of depths the parallax analysis generally reports a value close to the average depth.[4] Evidence in this report suggests another type of distribution must be considered, one in which the average location is governed by an equilibrium between fluorophore molecules at two distinct depths. To understand how this will affect depth measurements, the relationship between measured z_{cf} and the distribution of a fluorophore at two different depths must be derived.

Assume that the quenching of a fluorophore in one plane of a bilayer by a quencher in a different plane is given by (*5*)

$$F/F_o = \exp(-\pi R_c^2 + \pi z^2)C \qquad z < R_c \tag{1}$$

$$F/F_o = 1 \qquad \text{if } z > R_c \tag{2}$$

where F is the fluorescence in the presence of quencher, F_o is the fluorescence in the absence of quencher, R_c is the critical quenching distance, z is the distance between the fluorophore and quencher-containing planes (i.e., difference in their depth), and C is the quencher concentration (*5*). If a fluorophore is present at two different depths, then fluorescence is given by

$$F/F_o = (1-\alpha)[\exp(-\pi R_c^2 + \pi z_{f1}^2)C] + \alpha[\exp(-\pi R_c^2 + \pi z_{f2}^2)C] \tag{3}$$

$$= (1-\alpha)[\exp(-\pi R_c^2 + \pi z_{f1}^2)C] + \alpha \qquad \text{if } z_{f2} > R_c \tag{4}$$

$$= (1-\alpha) + \alpha[\exp(-\pi R_c^2 + \pi z_{f2}^2)C] \qquad \text{if } z_{f1} > R_c \tag{5}$$

where z_{f1} and z_{f2} refer to the difference in depth between a quencher and shallower and deeper fluorophore populations, respectively, and α is the fraction of fluorophores at the deeper depth (more rigorously α is the fraction of fluorescence arising from the deeper fluorophores in the absence of quencher).

To solve for the effect of having two fluorophore populations on measured depth, F/F_o values were calculated from eqs 3−5 (as appropriate) for cases with different proportions of fluorophores at two defined depths and in which quenching by TempoPC or 5SLPC is measured. The F/F_o values were then substituted into the parallax equation (see Experimental Procedures) and z_{cf} values were calculated (F/F_o with TempoPC being F_1 in this case and F/F_o with 5SLPC being

[4] We previously demonstrated that if there is an even distribution of quenchers and fluorophores over a range of depths then the measured z_{cf} should be very close to average z_{cf} (*6*). This should also be true for a Gaussian or any other symmetric distribution of molecules over a range of depths. Any such distribution can be represented as the sum of subdistributions with an even distribution between various upper and lower ranges of depth. Because for *any* one of these subdistributions measured z_{cf} will be close to the average z_{cf}, this should also be true for the full distribution.

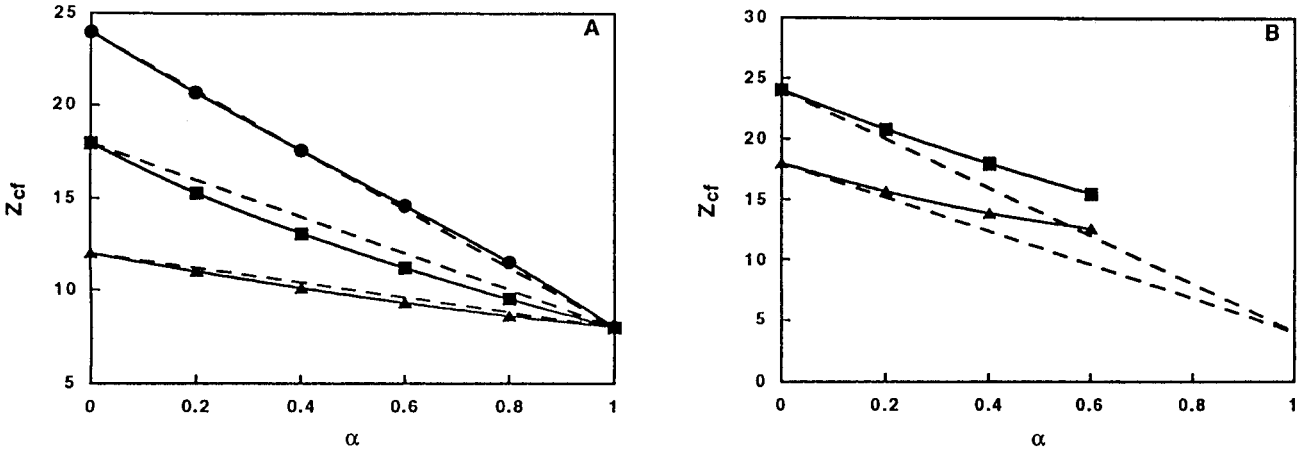

FIGURE 4: The effect of having two populations of a fluorophore on the depth calculated by the parallax analysis. (A) The effect of different fractional distributions of a fluorophore on calculated z_{cf} when both fluorophore populations are at depths $< R_c$ away from quencher depth. Measured z_{cf} is shown as solid lines for shallow populations at (●) 24 Å, (■) 18 Å, or (▲) 12 Å from the bilayer center and a deep population 8 Å from the bilayer center. (B) The effect of different fractional distributions of a fluorophore on calculated z_{cf} when one fluorophore population is at a depth $> R_c$ away from quencher depth. Measured z_{cf} is shown as solid lines for shallow populations at (■) 24 Å or (▲) 18 Å from the bilayer center and a deep population 4 Å from the bilayer center. Quenching was calculated for the case where TempoPC and 5SLPC are the quenchers, 15% of the lipid carries the quencher, and R_c is 12 Å. α is the fraction of fluorophores at the deep location. The true average z_{cf} for each case is shown as a dashed line (see Appendix for details).

F_2. Notice that in the parallax equation, the subscripts 1 and 2 refer to shallower and deeper quenchers, not fluorophores.) The dependence of measured z_{cf} on the two fluorophore depths and fractional distributions of fluorophores at each of these depths must be compared to the true average z_{cf} given by

$$z_{cf}\ \text{average} = (1 - \alpha)z_{cf1} + \alpha z_{cf2} \qquad (6)$$

where z_{cf1} and z_{cf2} are the distances of each fluorophore population from the center of the bilayer.

One case that can be analyzed is when shallower and deeper fluorophores are close enough to the quenchers in depth that the exponential form of the quenching equation (eq 3) is valid for both quenchers [i.e., the distance between fluorophore and both quenchers is less than the critical quenching distance, R_c (*5*)]. Figure 4A shows that in this case measured z_{cf} will be a function of the fraction of fluorophores at each depth and will be very close to (within 1 Å) to the true average z_{cf} (eq 6).

Of more concern is the case where one fluorophore population is so distant in depth from one of the quenchers being used that the partly nonexponential eqs 4 or 5 apply. This leads to an additional difference between measured z_{cf} using eq 1 and the true average z_{cf}, since the parallax equation assumes the exponential form of the quenching (equation 1) is valid, and this is only true for the population of fluorophores closest to the quencher. In Figure 4B an example of this case is shown where the predominant form of the fluorophore is shallow, and the TempoPC/5SLPC pair is used to calculate depth, but the secondary deep population is at a depth beyond R_c from TempoPC depth. In this case, Figure 4B shows measured z_{cf} is still close to average z_{cf} unless the secondary population is very large (>30%). When the secondary population is large, z_{cf} is biased toward that of the fluorophore population closest to the quenchers. Therefore, the error due to a secondary population is serious when the secondary population is large and of very different depth than the primary population. Nevertheless, it must be emphasized that z_{cf} represents only an approximate average value when there is a complex distribution of fluorescent groups over a range of depths.

Even more realistic models of quenching would assume a distribution of fluorophores around both the deeper and shallower fluorophore locations. However, since Gaussian and other symmetric fluorophore distributions tend to yield average depth (6), this should not strongly alter the results obtained.[4] Another more realistic model would also have a dependence of F/F_o on fluorophore and quencher depth is more complicated than given in eqs 1 and 2, because as z approaches R_c, these equations only give a crude estimate of the amount of quenching (*5*). This would have the effect of somewhat increasing the difference between measured and true average z_{cf}. Further analysis of such situations must await the determination of the distance dependence of quenching at higher resolution.

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