Exclusion of a Transmembrane-Type Peptide from Ordered-Lipid Domains (Rafts) Detected by Fluorescence Quenching: Extension of Quenching Analysis to Account for the Effects of Domain Size and Domain Boundaries†

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ABSTRACT: Sphingolipid/cholesterol-rich rafts are membrane domains thought to exist in the liquid-ordered state. To understand the rules governing the association of proteins with rafts, the behavior of a model membrane-inserted hydrophobic polypeptide (LW peptide, acetyl-$K_2W_2L_8AL_8W_2K_2$-amide) was examined. The distribution of LW peptide between coexisting ordered and disordered lipid domains was probed by measuring the amount of LW Trp fluorescence quenched by a nitroxide-labeled phospholipid that concentrated in disordered lipid domains. Strong quenching of the Trp fluorescence (relative to quenching in model membranes lacking domains) showed that LW peptide was concentrated in quencher-rich disordered domains and was largely excluded from ordered domains. Exclusion of LW peptide from the ordered domains was observed both in the absence and in the presence of 25−33 mol % cholesterol, indicating that the peptide is relatively excluded both from gel-state domains (which form in the absence of cholesterol) and from liquid-ordered-state domains (which form at high cholesterol concentrations). Because exclusion was also observed when ordered domains contained sphingomyelin in place of DPPC, or ergosterol in place of cholesterol, it appeared that this behavior was not strongly dependent on lipid structure. In both the absence and the presence of 25 mol % cholesterol, exclusion was also not strongly dependent upon the fraction of the bilayer in the form of ordered domains. To evaluate LW peptide behavior in more detail, an analysis of the effects of domain size and edges upon quenching was formulated. This analysis showed that quenching can be affected both by domain size and by whether a fluorescent molecule localized at domain edges. Its application to the quenching of LW peptide indicated that the peptide did not preferentially reside at the boundaries between ordered and disordered domains.

In many eukaryotic cell membranes, it is now believed that domains rich in unsaturated phospholipids coexist with lipid rafts, liquid-ordered domains that are rich in both relatively saturated sphingolipids and sterols. The association of proteins with rafts has been implicated as essential for a variety of cellular processes (*1*−*7*). Thus, understanding the principles that govern how lipids and proteins associate with rafts is important for understanding raft function. Some of these principles have been defined for lipid−lipid interaction. In particular, tight packing between relatively saturated lipids and cholesterol is a key feature that stabilizes raft formation (*8*, *9*). When these lipids pack tightly, they form the Triton X-100-insoluble liquid-ordered state (*8*, *10*−*12*).

Certain principles governing the association of proteins with rafts have also been elucidated. Cell-derived detergent-resistant membranes, which are believed to arise from rafts, are especially enriched in proteins anchored to membranes by saturated acyl chains (*13*, *14*). Strong association of such proteins with detergent-insoluble sphingolipid/cholesterol-rich domains has also been demonstrated using model membranes (*11*, *15*−*17*). These observations have been explained by the ability of tightly packed, ordered-lipid domains to accommodate saturated-lipid anchors (*11*). In contrast, covalently linked prenyl groups, which have structures that should prevent their packing tightly within ordered-lipid domains, are not likely to contribute to the association of proteins with rafts (*14*, *18*, *19*).

In general, transmembrane (TM)[1] proteins also would not be expected to have the ability to pack tightly with lipids in ordered domains and thus should be excluded from lipid rafts. A lack of detergent insolubility has been used to show that the TM protein bacteriorhodopsin is excluded from liquid-ordered domains *in vitro* (*15*). In addition, Van Duyl et al. recently demonstrated by detergent solubility that a hydrophobic peptide with an alternating Leu−Ala sequence was

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[1] Abbreviations: DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPH, diphenylhexatriene; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; LW peptide, acetyl-$K_2W_2L_8AL_8W_2K_2$-amide; MLV, multilamellar vesicles; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; PBS, phosphate buffered saline; 12SLPC, 1-palmitoyl-2-(12-doxyl)stearoyl-*sn*-glycero-3-phosphocholine; rhodamine-PE, *N*-(lissamine rhodamine B)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; SM, sphingomyelin; TM, transmembrane.

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excluded from ordered domains regardless of the extent of any hydrophobic mismatch between bilayer width and polypeptide length (*20*).

Spectroscopic approaches avoiding potential artifacts arising from detergent-based methods are also valuable for such studies. In bilayers lacking cholesterol, fluorescence quenching by nitroxide-labeled lipids has been used to show that several different polypeptides are excluded from ordered-gel phases (*21*−*23*). In a notable energy transfer study, Polozova and Litman found that at high cholesterol levels rhodopsin is depleted from ordered domains and enriched in disordered domains containing a highly polyunsaturated lipid (having 22:6 acyl chains) (*24*).

Nevertheless, some TM proteins are believed to associate with lipid rafts (*25*, *26*). In certain cases, the binding of such proteins to gangliosides, which are believed to be raft-associated, may result in raft association (*27*, *28*). In other cases, covalent linkage of TM proteins to saturated acyl chains is necessary for raft association (*14*, *29*−*35*). However, attachment to saturated acyl chains may not be the only factor in the raft association of such proteins. For example, caveolin-1 associates with ordered domains, yet mutations that prevent the attachment of saturated acyl chains to it do not prevent its association with rafts (*36*). In addition, changes in the amino acid sequence of TM segments in influenza hemagglutinin can abolish raft association without altering palmitoylation (*37*), and a quenching study suggested that a hydrophobic mismatch may affect the partition of gramicidin A′ between gel and fluid phases (*38*).

In this paper, we investigated the behavior of a simple 25-residue hydrophobic peptide (LW peptide) to explore the interaction, or lack of interaction, of membrane proteins with rafts. To provide a strong fluorescence signal, LW peptide was designed with two Trp at the N-terminal boundary plus two Trp at the C-terminal boundary of its hydrophobic sequence. LW peptide is a member of the well-studied polyLeu class of peptides. This class of polypeptides forms stable TM helices (*39*−*43*). The distribution of LW peptide between domains was determined by measuring its intrinsic Trp fluorescence when it was inserted into model membranes containing both disordered domains that were enriched in a fluorescence quenching lipid and quencher-depleted ordered domains. These experiments revealed that LW peptide was relatively excluded from ordered domains under a variety of conditions.

Lipid domain size can be drastically dependent on the exact experimental conditions (*44*), and rafts may be extremely small in cells (*45*, *46*). Therefore, we also analyzed the impact of domain/raft size on quenching. This analysis showed that the quenching method should both be able to detect very small rafts and be able to determine whether molecules concentrate at raft boundaries. Our study indicates that fluorescence quenching should be a useful way to systematically investigate the relationship between the sequence/structure of TM proteins and the nature of their association with lipid rafts.

EXPERIMENTAL PROCEDURES

Materials. Dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), brain sphingomyelin, 1-palmitoyl-2-(12-doxyl)stearoylphosphatidylcholine (12SLPC), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Androstenol was purchased from Sigma Chemical (St. Louis, MO). Ergosterol was purchased from Fluka Chemical (Ronkonkoma, NY). NBD-PE and rhodamine-PE were purchased from Molecular Probes (Eugene, OR) or Avanti Polar Lipids. LW peptide, acetyl-$K_2W_2L_8AL_8W_2K_2$-amide, was purchased from Research Genetics, Inc. (now Invitrogen, Carlsbad, CA). It was purified by reverse-phase high-pressure liquid chromatography using an 2-propanol/water gradient as described previously (*47*). A fraction (about 20%) of the purified peptide molecules contained a one Leu deletion. The interaction of this slightly shorter peptide with lipid is likely to be very similar to that of the full-length LW peptide. Diphenylhexatriene (DPH) was purchased from Sigma-Aldrich (St. Louis, MO). Lipid concentrations were determined by dry weight, and peptide concentrations were determined by spectrophotometry using a molar absorptivity value at 280 nm of 5560 M^{-1} cm^{-1} per Trp residue. Lipids, DPH, and peptide were dissolved in ethanol and stored at −20 °C until use.

Preparation of Multilamellar Vesicles (MLV). Ethanolic solutions of lipids and peptide were allowed to warm to room temperature for 1 h prior to use. Samples were prepared by mixing appropriate volumes of lipids and peptide or DPH into 12 × 75 mm disposable glass tubes, using calibrated glass capillary pipets. After mixing, the samples were dried under N_2, after which 20 μL of chloroform was added to each tube. Samples were then redried, first with N_2 and then under high vacuum for 1 h. Samples containing DPH were covered to protect them from light during the drying processes. To form MLV, 800 μL of PBS (10 mM Na phosphate, 150 mM NaCl, pH 7.4), warmed to 50−55 °C, was added to each sample. They were then immediately placed into a water bath (at 50−55 °C), covered, and incubated for 10 min. The samples were transferred to a multi-tube vortexer (VWR Scientific, West Chester, PA), placed in a 50−55 °C chamber, and agitated vigorously for 2−4 min. The samples were removed, briefly individually vortexed while exposed to room temperature, and finally allowed to cool. In rare cases in which the lipids did not disperse well after vortexing, samples were sonicated for 5−10 s in a bath sonciator (model G112SP1T, Laboratory Supplies, Hicksville, NY). Prior to measurements, samples were incubated at room temperature for at least an additional 45 min. Samples containing DPH were covered to protect them from light during this period.

Preparation of Ethanol Dilution Vesicles. Ethanol dilution vesicles were prepared similarly to MLV, except that after the initial drying with N_2, 16 μL of ethanol was added to redissolve the samples. Next, 784 μL of PBS warmed to 50−55 °C was added to each sample, and they were then placed in a 50−55 °C bath for 5 min. As above, the samples were briefly vortexed manually and then allowed to cool to room temperature prior to fluorescence measurements.

Fluorescence Quenching Experiments. Vesicle samples for thermal scanning experiments contained 50 μM total lipid (phospholipids plus sterol) plus 0.5−2 mol % peptide and/or 0.25 mol % DPH. Unless otherwise noted, these samples were prepared by the MLV procedure described previously. In most cases, DPPC- or SM-containing samples with quencher (*F* samples) contained a 2:1:1 (mol/mol) [DPPC

or SM]/DOPC/12SLPC mixture with or without 25 mol % sterol. Corresponding samples without quencher (F_o samples) contained 1:1 DPPC/DOPC with or without 25 mol % sterol. In other experiments, lipid compositions of 3:1 mol:mol DPPC/12SLPC and F_o samples of 3:1 DPPC/DOPC were used. For both the 2:1:1 and the 3:1 compositions, control samples, which contained quencher but which could not form domains, were also prepared. These contained a 3:1 mixture of DOPC/12SLPC with or without 25 mol % sterol. Corresponding control F_o samples contained DOPC with or without 25 mol % sterol. For each lipid composition, background samples were prepared identically, except without peptide or DPH. Samples containing peptide or DPH were generally prepared in quadruplicate. (In some cases, DPH samples were prepared in duplicate.) Fluorescence in each sample was measured at 23 °C to assess sample-to-sample variation, and the quadruplicates were then pooled. The fluorescence in pooled samples was then measured at a series of increasing temperatures as described next. Pooling samples was found to significantly reduce the variability of fluorescence intensities observed relative to that when individually prepared samples were used. Prior to pooling the samples, the standard deviations of the fluorescence intensities for the four individual samples generally ranged between 5 and 10% of the average F or F_o values. In the unusual cases in which a larger variation was observed, the experiments were usually repeated with an additional set of pooled quadruplicates, and values for the two pooled sets were averaged. Background values were found to be largely temperature independent and so only monitored at the lowest (starting) temperatures. Background values for peptide experiments were subtracted from the sample fluorescence intensities before F/F_o values were calculated. In the case of DPH experiments, backgrounds had a negligible fluorescence intensity.

For experiments in which DPPC concentration was varied, ethanol dilution vesicles were prepared as described previously. Samples contained 50 μM total lipid (phospholipids plus sterol) and 2 mol % LW peptide dispersed in PBS. The sample volume was 800 μL. Samples, with or without 25 mol % cholesterol, contained various mixtures of DPPC and 12SLPC or DOPC and 12SLPC. Corresponding F_o samples contained DOPC in place of the 12SLPC. For each lipid composition, background samples were prepared identically, except without peptide. Their fluorescence was subtracted from the sample fluorescence intensities before F/F_o values were calculated. Fluorescence in each sample was measured at 23 °C.

Fluorescence Measurements. Fluorescence emission spectra were measured on a Spex 212 Fluorolog fluorimeter using 10-mm excitation path length, 4-mm emission path length semimicro quartz cuvettes. Peptide fluorescence was measured at an excitation wavelength of 280 nm and emission wavelength of 340 nm. DPH fluorescence was measured at an excitation wavelength of 359 nm and an emission wavelength of 427 nm. Excitation slits were set at 2.5 mm (4.5-nm band-pass) for peptide or 1.25 mm for DPH. Emission slits were set at 5.0 mm. Temperatures were adjusted using a variable temperature water bath connected to a sample holder through which the bath solution circulated. When desired, sample temperatures were monitored with an electronic thermometer using a narrow probe placed within the sample solution, and the fluorescence was read 2 min after the samples reached the desired temperature.

Sucrose Gradient Centrifugation. Sucrose gradient centrifugation was used to analyze the behavior of vesicles prepared by ethanol dilution. One preparation of vesicles contained a 100 μM 1:1 DPPC/DOPC or 3:3:2 DPPC/DOPC/cholesterol (mol/mol) mixture plus 5 mol % LW peptide and 0.25 mol % rhodamine-PE. It was dispersed in 2.5 or 10% (w/v) sucrose. A second preparation of vesicles lacking peptide contained 100 μM 1:1 DPPC/DOPC or 3:3:2 DPPC/DOPC/cholesterol plus 0.5 mol % NBD-PE. It was also dispersed in 2.5 or 10% (w/v) sucrose. (In other experiments, similar samples with or without peptides were prepared with the DPPC replaced by an equivalent amount of DOPC.) After 400 μL of the peptide-containing and peptide-lacking preparations were mixed, the external sucrose concentration was increased to 20% (w/v) with solid sucrose. The samples were then loaded on a 100 μL cushion of 50% (w/v) sucrose and overlayed with 3.6 mL of a 10−0% (w/v) linear sucrose gradient. Samples were centrifuged for 17 h at 38 000 rpm in a Beckman ultracentrifuge using a SW 60 swinging bucket rotor. Fractions of 200 μL were removed sequentially from the bottom of the tube. Trp fluorescence in each fraction was measured at an excitation wavelength of 280 nm and emission wavelength of 340 nm, NBD fluorescence was measured at an excitation wavelength of 470 nm and emission wavelength of 530 nm, and rhodamine fluorescence was measured at an excitation wavelength of 565 nm and emission wavelength of 585 nm. Control vesicle samples with and without each fluorophore showed that there was no significant contribution of any fluorophore to the background fluorescence intensity for any other fluorophore.

RESULTS

Quenching Analysis of the Partitioning of a Fluorescent Molecule between Ordered and Disordered Domains in Lipid Bilayers. Both domain formation and polypeptide association with specific lipid domains were assayed using fluorescence quenching ((*9*, *10*, *18*, *21*, *48*, *49*)). In this approach, the partitioning of a fluorescent molecule is probed by the use of lipid vesicles containing a mixture of ordered and disordered domains. The vesicles contain lipids such as DPPC or sphingomyelin (SM), both of which tend to form ordered domains at room temperature, and 12SLPC, a lipid that carries a fluorescence quenching nitroxide group and that prefers to form and/or partition into disordered domains (*10*). Because of 12SLPC-induced quenching, fluorophores residing within 12SLPC-rich disordered domains fluoresce weakly, whereas those in the ordered domains, which are 12SLPC-poor, fluoresce more strongly (*21*, *38*, *50*).

Experimentally, both the presence of domains and the partitioning of a fluorescent molecule between ordered and disordered domains are assessed by comparing quenching in samples containing lipid domains to quenching in analogous control lipid mixtures that form homogeneous bilayers lacking domains. It has been previously shown that a molecule associating to a significant degree with 12SLPC-depleted ordered domains is quenched less, and thus exhibits stronger fluorescence, in samples containing a mixture of ordered and disordered domains than in a homogeneous bilayer containing the same amount of 12SLPC (*9*, *10*, *49*).

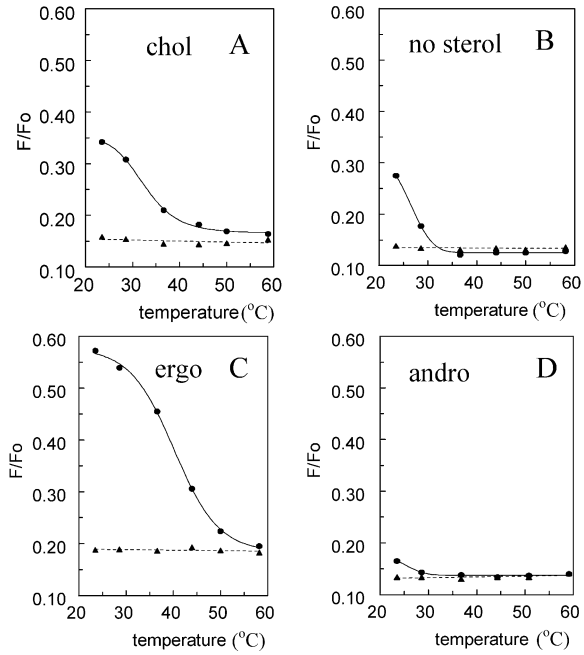

FIGURE 1: Quenching of DPH fluorescence in lipid mixtures containing or lacking ordered domains and various sterols. Quencher-containing vesicles were composed of DPH and (circles) 2:1:1 DPPC/DOPC/12SLPC (mol/mol) or (triangles) 3:1 DOPC/12SLPC with (A) 25 mol % cholesterol, (B) no sterol, (C) 25 mol % ergosterol, or (D) 25 mol % androstenol. Samples contained MLV at a concentration of 50 μM lipid with 0.25 mol % DPH and were dispersed in PBS. Samples were heated, and at each temperature the fluorescence in quencher (12SLPC)-containing vesicles (F) and in quencher-free (F_o) vesicles, in which 12SLPC was replaced by DOPC, was measured. F/F_o values were calculated after subtraction of the fluorescence in background samples lacking the fluorophore.

In contrast, a molecule residing only in the 12SLPC-rich disordered domains is quenched more strongly in a sample containing a mixture of domains than in a homogeneous bilayer. A more detailed discussion has been presented elsewhere (*9*, *10*, *18*, *21*, *48*, *49*).

Domain Formation in DPPC-Containing Bilayers. Preliminary studies (not shown) indicated that the partitioning of LW peptide between ordered and disordered domains could be assessed with maximal sensitivity in a mixture of 2:1:1 (mol/mol) DPPC/DOPC/12SLPC. At room temperature, we found previously that this mixture forms bilayers containing nearly equal amounts of DPPC-rich ordered domains and 12SLPC/DOPC-rich disordered domains (*10*). However, we had not examined the behavior of this lipid composition under the wide range of conditions used in the studies described next. To do so, we evaluated quenching of DPH fluorescence. (DPH is useful for such experiments because it partitions about equally well between most types of ordered or disordered domains and so will not perturb domain formation (*21*, *51*).) Fluorescence quenching was assessed from the parameter F/F_o, which is the ratio of fluorescence in samples containing quencher (12SLPC) to that in samples in which quencher is replaced by DOPC. A low F/F_o value corresponds to a high degree of quenching.

Figure 1 illustrates the quenching of DPH fluorescence in multilamellar vesicles (MLV) formed from 2:1:1 DPPC/DOPC/12SLPC mixtures with or without 25 mol % sterol. In each case, higher F/F_o values were observed in the DPPC-containing mixtures (circles) than in control mixtures containing DOPC and 12SLPC (triangles) at 23 °C. The control mixtures form bilayers in a homogeneous disordered fluid state over the entire temperature range used (*9*, *10*). Thus, the weak quenching of DPH fluorescence in the DPPC-containing samples relative to control samples confirmed that the former contained domains at low temperature. At higher temperatures, quenching in the DPPC-containing samples increased to levels equivalent to that in the control samples, showing that domains in the DPPC-containing samples disappeared. High-temperature abolishes domains due to the melting of ordered domains and the mixing of their lipids with those in the disordered domains (*9*). Combined with previous studies of similar lipid mixtures (*9*), these results showed that at low temperatures DPPC-containing samples contained DPPC-rich ordered domains in equilibrium with DOPC/12SLPC-rich disordered domains.

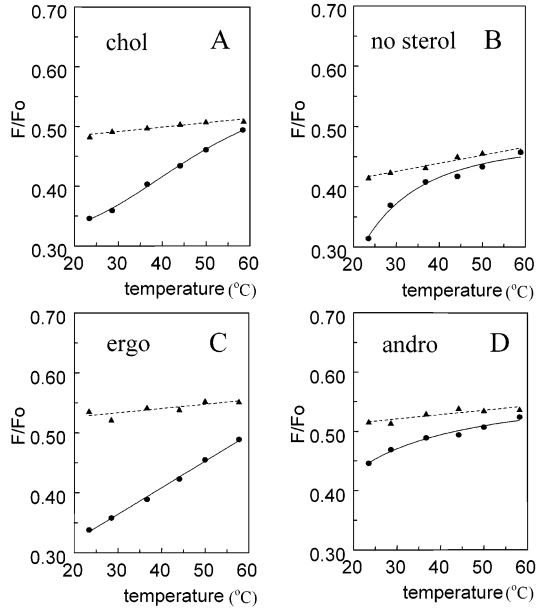

FIGURE 2: Quenching of LW peptide fluorescence in lipid vesicles containing or lacking ordered domains and various sterols. Quencher-containing vesicles were composed of LW peptide and (circles) 2:1:1 DPPC/DOPC/12SLPC (mol/mol) or (triangles) 3:1 DOPC/12SLPC with (A) 25 mol % cholesterol, (B) no sterol, (C) 25 mol % ergosterol, or (D) 25 mol % androstenol. Samples contained MLV at a concentration of 50 μM lipid with 2 mol % LW peptide and were dispersed in PBS. Experiments were performed as described in Figure 1.

The difference between quenching in the DPPC-containing and control mixtures illustrated in Figure 1 also showed that the thermal dependence of quenching was significantly affected by the presence of 25 mol % sterol. Relative to bilayers lacking sterol (Figure 1B), in the presence of cholesterol and ergosterol there was less quenching at 23 °C and a higher temperature for the transition from a domain-containing (weaker quenching) to a domain-lacking (stronger quenching) state (Figure 1A,C). Overall, the degree of ordered domain formation at 23 °C and the thermal stability of ordered domains decreased in the order ergosterol > cholesterol > no sterol > androstenol. This ranking is in good agreement with that found previously in similar lipid mixtures containing 15 mol % sterol (*9*, *52*).

Partitioning Behavior of LW Peptide between Ordered and Disordered Domains: Effect of Sterol. Next, quenching was used to study the behavior of LW peptide. Figure 2 shows

the temperature dependence of the fluorescence quenching of LW peptide incorporated into MLV having the same compositions as in the DPH studies described previously. In all cases, at 23 °C, F/F_o was lower in DPPC-containing domain-forming samples (circles) than in the homogeneous control samples that lacked DPPC (triangles). As the temperature was increased, quenching in the DPPC-containing samples decreased, finally reaching values close to that in the control samples at 60 °C. As in the case of the quenching of DPH, this change in quenching is due to the melting of the ordered domains in the DPPC-containing samples at high temperature, which induces a state of homogeneous lipid mixing similar to that in the control sample. In experiments in which DPPC-containing samples were cooled to room temperature, this change was found to be largely reversible (not shown). As expected, control samples exhibited at most a very weak dependence of quenching upon temperature.

The stronger quenching of LW peptide in DPPC-containing bilayers relative to control samples indicated that the LW peptide partitioned favorably into disordered domains and was relatively depleted from the ordered domains. Previous studies indicate that the ordered domains in lipid mixtures containing cholesterol are cholesterol-rich and exist in the liquid-ordered state (*10*, *11*). Ordered domains forming in the absence of cholesterol are in the gel state. Therefore, we conclude that the LW peptide was excluded from both liquid-ordered and gel-state domains.

It is noteworthy that quenching of LW peptide in the absence or presence of various sterols largely mirrored the level of ordered-domain formation. This was shown by the observation that the differences between F/F_o in the DPPC-containing (circles) and control samples (triangles) decreased in an order (ergosterol ≥ cholesterol > no sterol ≥ androstenol) similar to that observed for DPH quenching. This pattern suggests that LW peptide behavior was influenced by sterol effects on domain formation rather than specific sterol−peptide interactions.

Despite the parallels between the quenching patterns for DPH and LW peptide, it was apparent that the curves describing the temperature dependence of quenching LW peptide showed more gradual changes in F/F_o than for DPH. This indicated that the thermal process sensed by LW quenching was more gradual in terms of its temperature dependence than that sensed by DPH (see next and Discussion).

It was also noted that F/F_o values for LW peptide in homogeneous bilayers in the absence of sterol (Figure 2B, triangles) were lower than in homogeneous samples containing sterol (Figure 2A,C,D, triangles) under equivalent conditions. The stronger quenching in the absence of sterol probably resulted from the fact that in the absence of sterol, 12SLPC molecules made up a higher fraction of the total lipid and so had a higher concentration within the bilayer.

Strong Association of LW Peptide with Disordered Domains Is Observed under a Range of Conditions. These findings were next expanded by evaluating the partitioning of LW peptide under a variety of conditions. Both domain formation (as detected by DPH fluorescence, Figure 3D,E) and the preferential association of LW peptide with disordered domains (Figure 3A,B) were maintained when the amount of cholesterol was increased to 33 mol % (Figure 3A,D) or in small unilamellar vesicles prepared by ethanol dilution (Figure 3B,E). Other experiments showed that LW peptide also associated with disordered domains when DPPC was replaced by sphingomyelin (SM), a natural component of lipid rafts with phase behavior similar to that of DPPC (*10*) (Figure 3C). It should be noted that a thermal dependence of the quenching of the LW peptide similar to that observed with 2 mol % peptide was observed in DPPC-containing samples with 25 mol % cholesterol when the concentration of peptide in the bilayer was decreased to 0.5 mol % (data not shown).

Since LW peptide preferentially associates with disordered domains, it might be expected to destabilize ordered-domain formation. However, the DPH quenching showed that ordered-domain formation in DPPC-containing mixtures in the presence of 2 mol % LW peptide (Figure 3F) was not markedly different than that in its absence (Figure 1A). The inability of LW peptide to affect domain formation may stem from the fact that these samples had a relatively small amount of peptide relative to lipid in disordered domains. When the amount of lipid in disordered domains is small or polypeptide concentration is high, polypeptides may have much more dramatic effects upon domain formation (see next) (*24*).

Effect of the Fraction of Ordered Lipid on the Partition Behavior of LW Peptide. The effect of varying the mol fractions of DPPC and 12SLPC upon the quenching of LW peptide fluorescence was examined because under favorable conditions a profile of fluorescence versus DPPC mol fraction can be used to calculate K_p, the partition coefficient (*21*). This parameter is equal to the ratio of LW peptide concentration in disordered domains to that in ordered domains. The difference between quenching in DPPC/12SLPC mixtures and DOPC/12SLPC control mixtures depends on the value of K_p (*10*, *21*) and should be observed throughout the range of lipid compositions over which ordered and disordered domains coexist. Previous studies have shown that in DPPC/12SLPC mixtures at 23 °C, bilayers are fully in the ordered state above 80 mol % DPPC (i.e., below 20 mol % 12SLPC), fully in the disordered state below 20 mol % DPPC (i.e., above 80 mol % 12SLPC), and contain coexisting ordered and disordered domains between 20 and 80 mol % DPPC (= 80−20 mol % 12SLPC). This is true both in the presence and in the absence of cholesterol (*10*). Therefore, K_p-dependent quenching is expected in samples containing between 20 and 80 mol % 12SLPC.

Figure 4 shows the effect of the 12SLPC mol fraction upon quenching of LW peptide fluorescence in vesicles prepared by ethanol dilution. LW peptide exhibited stronger quenching in DPPC/12SLPC mixtures (Figure 4, closed squares) than in the corresponding homogeneous DOPC/12SLPC control mixtures (Figure 4, open squares) over the entire 20−80 mol % 12SLPC range. This was true both in the absence (Figure 4A) and in the presence (Figure 4B) of cholesterol. These results showed that LW peptide strongly preferred to partition into disordered domains over the entire range of domain coexistence (i.e., that its preference for disordered domains did not strongly depend on what fraction of the bilayer was in the form of disordered domains).

Theoretical curves describing the dependence of quenching upon K_p, calculated as described previously (*21*), are illustrated in Figure 5A. Comparison of Figures 4A to 5A

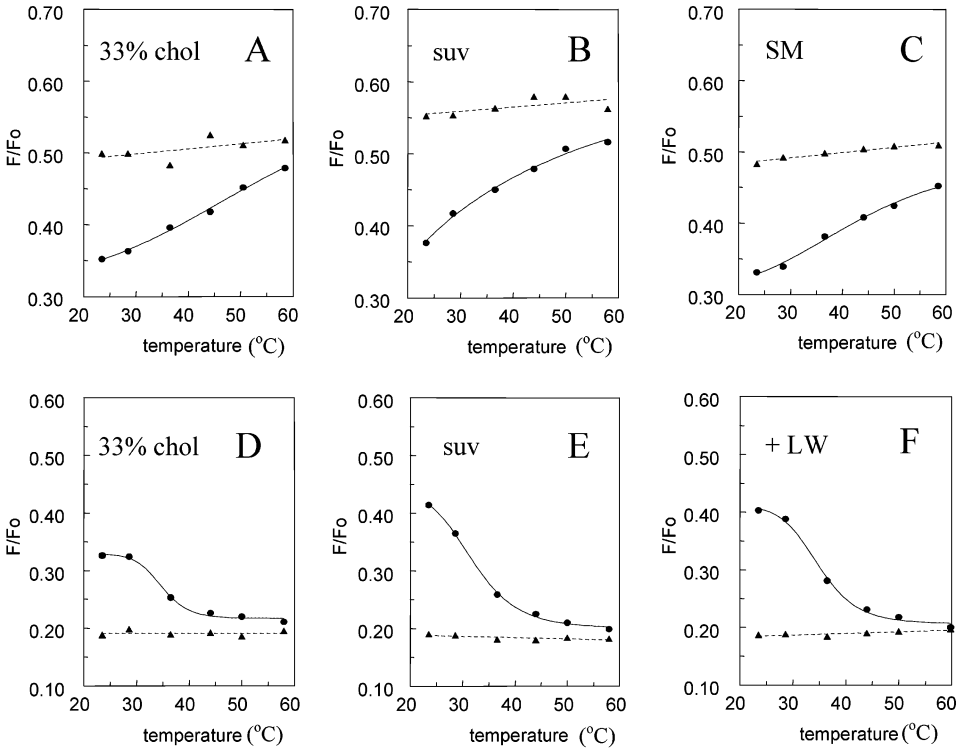

FIGURE 3: Quenching of LW peptide and DPH fluorescence in lipid mixtures containing or lacking ordered domains under various conditions. (A) Quenching of LW peptide fluorescence in samples containing 33 mol % cholesterol. Quencher-containing vesicles were composed of 2 mol % LW peptide and (circles) 2:1:1:2 DPPC/DOPC/12SLPC/cholesterol or (triangles) 3:1:2 DOPC/12SLPC/cholesterol. (B) Quenching of LW peptide fluorescence in vesicles prepared by ethanol dilution. Quencher-containing vesicles were composed of 2 mol % LW peptide and (circles) 2:1:1:1.33 DPPC/DOPC/12SLPC/cholesterol or (triangles) 3:1:1.33 DOPC/12SLPC/cholesterol. (C) Quenching of LW peptide fluorescence in quencher-containing vesicles containing SM. Vesicles contained 2 mol % LW peptide and (circles) 2:1:1:1.33 SM/DOPC/12SLPC/cholesterol or (triangles) 3:1:1.33 DOPC/12SLPC/cholesterol. (D) Quenching of DPH fluorescence in samples containing 33 mol % cholesterol. Quencher-containing vesicles were composed of 0.25 mol % DPH and (circles) 2:1:1:2 DPPC/DOPC/12SLPC/cholesterol or (triangles) 3:1:2 DOPC/12SLPC/cholesterol. (E) Quenching of DPH fluorescence in vesicles prepared by ethanol dilution. Quencher-containing vesicles were composed of 0.25 mol % DPH and (circles) 2:1:1:1.33 DPPC/DOPC/12SLPC/cholesterol or (triangles) 3:1:1.33 DOPC/12SLPC/cholesterol. (F) Quenching of DPH fluorescence in vesicles also containing LW peptide. Quencher-containing vesicles were composed of 0.25 mol % DPH and (circles) 2:1:1:1.33 DPPC/DOPC/12SLPC/cholesterol plus 2 mol % LW peptide or (triangles) 3:1:1.33 DOPC/12SLPC/cholesterol with 2 mol % LW peptide. Other experimental conditions and procedures are as in Figures 1 and 2.

suggests that the K_p for LW peptide is about 10−20. That is, there is a 10−20-fold higher concentration of LW peptide in disordered domains than in ordered gel domains. Comparison of Figure 4, panels B to A suggests that partition between ordered and disordered domains in the presence of cholesterol is similar to that in its absence. However, a more precise estimate of K_p in the presence of cholesterol is not possible because analysis of quenching behavior in a three-lipid component mixture (DPPC, 12SLPC, cholesterol) system is more complex than in a two-lipid component mixture (DPPC, 12SLPC) system, and K_p cannot be quantitatively modeled in the same manner (*18*).

It should be noted that LW peptide fluorescence intensity in the absence of quencher (F_o) tends to support the conclusion that LW peptide partitions strongly into the disordered domains. Figure 4C shows that F_o values in bilayers composed solely of DPPC with or without cholesterol were somewhat more than half of those composed of DOPC with or without cholesterol. (We did not explore the origin of this increase in intensity in the disordered domains, but a likely possibility is that, due to peptide insolubility in ordered domains, when disordered domains are absent the peptides aggregate laterally, and this induces Trp self-quenching (*53*). Self-quenching is likely to be abolished when the peptides are more dispersed in the disordered domains.) If LW peptide remained in ordered domains to an appreciable extent, then it would be predicted that F_o would only have increased gradually as a fraction of the bilayer in the disordered state was increased. Instead, the values of F_o increased rapidly as the DOPC concentration increased, reaching a value close to that in 100% DOPC at about 40 mol % DOPC. This is the behavior expected if the LW peptide partitioned strongly into DOPC-rich fluid domains, so that the LW peptide moved into disordered domains as soon as the DOPC concentration was high enough for an appreciable number of disordered domains to form.

Possible Evidence for Perturbation of Lipid Phase Behavior by LW Peptide. Interestingly, both with and without cholesterol, strong quenching of LW peptide in DPPC-containing bilayers was observed when bilayers contained only 10 mol % 12SLPC (Figure 4). Our previous data suggested that DPPC/12SLPC mixtures with 10 mol % 12SLPC formed a homogeneous ordered state (*10*). However, it was difficult to specify the exact lipid composition at which disordered domains began to form, and it was possible that a small amount of 12SLPC-rich disordered state domains were present when the bilayers contained 10 mol % 12SLPC. Thus, one explanation for the strong quenching of LW peptide in bilayers of this composition is that the LW peptide was concentrated in 12SLPC-rich domains. The somewhat

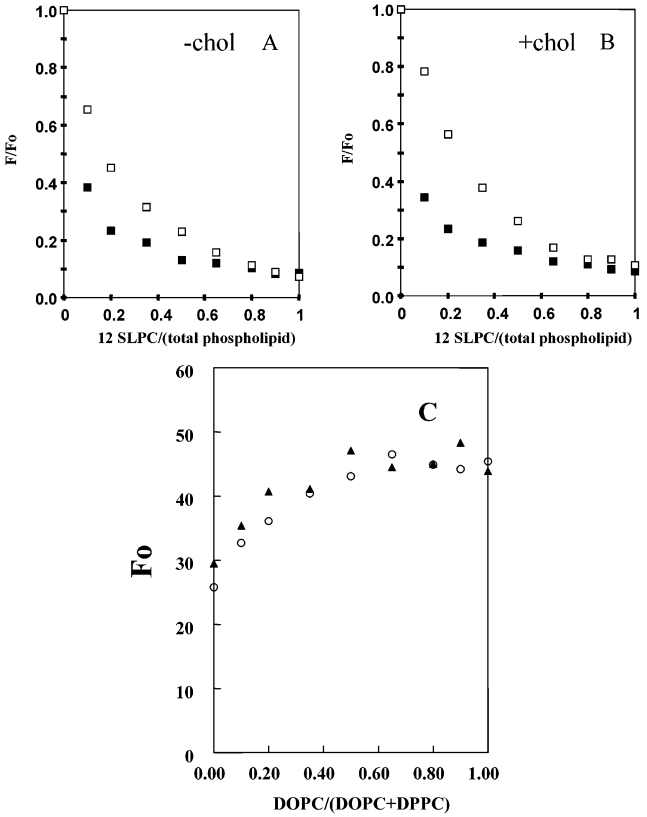

FIGURE 4: Quenching of LW peptide fluorescence as a function of lipid composition. Ethanol dilution vesicles were prepared with various amounts of 12SLPC and DPPC (closed squares) or 12SLPC and DOPC (open squares) (A) Vesicles without cholesterol. (B) Vesicles with 25 mol % cholesterol. Samples contained 2 mol % LW peptide and 50 μM total lipid dispersed in PBS. F/F_o was measured at room temperature as described in Figure 1. F_o samples contained DOPC in place of 12SLPC. The *x*-axis shows the concentration of 12SLPC as a mol fraction of the total phospholipid. (C) F_o values for DPPC-containing samples (those indicated by filled squares in panels A and B). (Triangles) F_o values for panel A; (circles) F_o values for panel B. The *x*-axis gives the fraction of DOPC as a mol fraction of the total phospholipid.

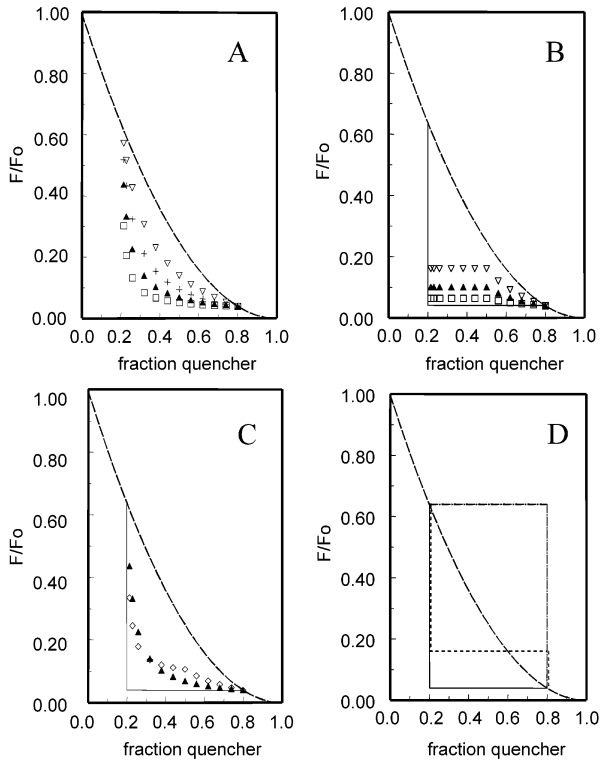

FIGURE 5: Calculated dependence of quenching of the fluorescence of a transmembrane helical polypeptide in bilayers composed of DPPC and 12SLPC. The fraction quencher is the fraction of the phospholipid molecules that are 12SLPC. In all four panels, F/F_o in a homogeneous bilayer that does not form domains is shown by long dashes. The other curves show F/F_o calculated using the equations derived in the Appendix for cases in which ordered domains contain 20 mol % 12SLPC, and disordered domains contain 80 mol % 12SLPC. (A) Effect of K_p upon quenching over the region of two-phase coexistence (compositions in which domains exist) for (open triangles) $K_p = 5$; (crosses) $K_p = 10$; (closed triangles) $K_p = 20$; and (squares) $K_p = 50$. (B) Effect of domain size upon quenching. It is assumed that the least abundant domains are small and are surrounded by a more abundant domain that is continuous, so that a shift between domains that are small occurs when equal amounts of ordered and disordered domains are present. Thus, for the case shown, disordered domains are small at the fraction 12SLPC < 0.5, while ordered domains are small at the fraction 12SLPC > 0.5. Quenching is shown in the two-phase range of compositions for K_p values that are near-infinite and in which the smaller domains have (solid line) infinite size so no molecules are near domain edges; (squares) 20% of molecules near the domain edge ($2x/w = 0.2$); (closed triangles) 50% of molecules near the domain edge ($2x/w = 0.5$); and (open triangles) 100% of molecules near the domain edge ($2x/w = 1$). (C) The effect of different combinations of K_p and domain size: (diamonds) quenching in the two-phase range of compositions for $K_p = 50$ and $2x/w = 0.5$ and (triangles) quenching in the two-phase range of the composition region for $K_p = 20$ and near-infinite domain size. (D) Effect of location of the fluorescent molecule for domains of near-infinite size. Quenching is shown for fluorescent molecules: (long dashes) in a homogeneous bilayer; (solid line) present only in disordered domains (i.e., when K_p = infinity); (dashes and dots) present only in ordered domains (i.e., when $K_p = 0$); and (short dashes) only associating with domain edges (i.e., K_{edge} = infinity).

weaker quenching at 10 mol % 12SLPC relative to that at higher mol % 12SLPC might be due to the fact that the quencher-to-peptide ratio is so low that there is not enough quencher lipid to fully surround the LW peptide molecules.

An alternative is that the LW peptide had such a strong propensity for association with disordered lipid domains that it nucleated the formation of 12SLPC-rich domains under conditions in which they would not exist in the absence of peptide. These domains might be as little as a 12SLPC-rich boundary layer of lipid molecules surrounding each LW peptide.

Effect of Domain Size Upon Fluorescence Quenching: Small Domain Size Can Prevent Calculation of Precise K_p Values. We were concerned that our interpretation of partition behavior might be incorrect if quenching was to be influenced by factors other than K_p, such as domain size. Domain size is important because small domains have relatively large boundary zones or edges. Unlike fluorescent molecules in the core of a domain, the fluorescence of molecules at the edge of a domain would be affected by quenchers both inside and outside the domain.

To define the influence of domain size on quenching, an analysis of the relationship between quenching and domain size was formulated (Appendix). The case in which the less abundant lipid phase forms small domains and the more abundant lipid phase forms a continuous phase that surrounds these small domains was analyzed. Figure 5B,C shows the predicted effects of domain size upon 12SLPC quenching for a fluorescent polypeptide. In Figure 5B, the case in which the fluorescent polypeptide is fully excluded from ordered

domains (i.e., for which the K_p is infinite) is considered. In this case, the qualitative effect of reducing the domain size down to very small dimensions is to reduce quenching relative to that observed with larger domains. For nitroxides, the quenching interaction distance is about equal to the diameter of a single lipid (*54*−*56*). As a result, a difference between nitroxide quenching in the presence of small domains (squares) relative to that in infinite size domains (solid line) would only become apparent when the domains have a width of <10 lipid molecules (see Appendix for details).

Interestingly, the effect of reducing domain size was found to depend to some degree upon whether the small domains were formed by the quencher-rich or -poor lipid phase. This is seen most easily by the change in the shape of the quenching curves in the presence of small domains (Figure 5B, triangles) at a 12SLPC mol fraction of 0.5. Notice that it might be difficult to distinguish bilayers containing extremely small ordered domains (triangles) from uniform bilayers (dashed line) under conditions in which the quencher-rich phase is predominant (at quencher fractions of 0.5−0.8 in Figure 5B).

Another consequence of the finding that quenching is affected by domain size is that it makes the determination of exact K_p values problematical. As shown in Figure 5C, it would be difficult to distinguish a case in which $K_p = 50$ and in which domains are very small, from that in which $K_p = 20$ and domains are large. Therefore, in the absence of an estimate of domain size, our experimental data allow us to state that the LW peptide strongly partitions into disordered domains but cannot be used to derive precise values for K_p.

Finally, it should be noted that the effect of decreasing domain size upon quenching depends on K_p. The effect of domain size upon quenching for a molecule that, like DPH, partitions equally between ordered and disordered domains (i.e., for which $K_p = 1$) is somewhat different than its effect on a molecule that, like the LW peptide, is excluded from ordered domains (see Appendix).

Effect of Specific Association of Fluorescence Molecules with Domain Edges upon Quenching: Evidence that LW Peptide Does Not Associate with Domain Edges. Another factor that could influence quenching is the location of a fluorescent molecule within individual domains. In particular, we analyzed how strongly quenching would be affected by whether a molecule preferentially located to domain edges (Appendix). On the basis of this analysis, quenching behavior was compared for a peptide located exclusively (1) within ordered domains, (2) within disordered domains, or (3) at domain boundaries (Figure 5D). Not surprisingly, quenching of a peptide located exclusively at domain boundaries (short dashes) was intermediate between that for one located exclusively within ordered domains ($K_p = 0$) (dashes and dots) and that for one located exclusively within disordered domains (K_p = infinity) (solid line). This analysis also revealed a unique feature of the quenching profile for fluorescent molecules associating with domain edges, namely, F/F_o values that cross the F/F_o curve for homogeneous bilayers (long dashes) at some 12SLPC concentration (Figure 5D). A crossing point of this type is not observed under other conditions, such as when a fluorescent molecule has an intermediate K_p value (Figure 5A−C).

Of course, a molecule might have partitioning behavior in which its behavior is characterized both by a K_p value and by a finite affinity for domain edges, defined as K_{edge}. Although we have not illustrated profiles for F/F_o with various combinations of K_p and K_{edge}, a crossing point would be expected for cases in which a molecule strongly favoring ordered domains over disordered domains, or vice versa, also had a very strong affinity for domain edges (calculation not shown). Thus, the experimental quenching data for LW peptide in Figure 4, which did not exhibit a crossing point, is not consistent with a significant degree of preferential LW peptide location at domain edges.

Origin of the Difference between Temperature Dependence of the Quenching of DPH and LW Peptide. As noted earlier, comparison of the data in Figure 1 to Figure 2 indicated that the thermal transition sensed by LW peptide was much more gradual than that sensed by DPH. We wished to further examine this phenomenon and its dependence of cholesterol. For the samples used in Figures 1 and 2 this was difficult because the thermal transition in samples lacking cholesterol occurred at too low a temperature to detect easily in our experimental setup. For this reason, we prepared new samples, in which the DPPC concentration was increased to 75 mol % of the total phospholipid. The increase in the DPPC concentration raised the melting temperature of ordered domains and thus made it easier to detect complete thermal melting/mixing curves.

Figure 6 shows the dependence of DPH and LW peptide quenching in such vesicles. Figure 6A shows that the melting of DPPC-enriched ordered domains, as monitored by DPH quenching, occurred with a midpoint about 5−10 °C higher in the presence of 25 mol % cholesterol (filled triangles) than in its absence (filled circles).[2] A similar effect of cholesterol on the transition midpoint was observed previously in samples containing 15 mol % cholesterol (*9*). Figure 6 also shows that cholesterol influences how sharply F/F_o changes with temperature both for DPH and for LW peptides. In the presence of cholesterol, the thermal transitions are more gradual than in its absence. This is not surprising, as it has been long known that from experiments in binary mixtures of cholesterol with DPPC that cholesterol reduces the thermal cooperativity of melting events (*57*).

Comparison of the behavior of LW peptide and DPH quenching shows that the thermal dependence of LW quenching in DPPC-containing samples exhibited a midpoint similar to that observed for DPH quenching in both the absence (Figure 6B) and the presence (Figure 6C) of cholesterol. However, in both cases, quenching changed more gradually with increasing temperature for the LW peptide than for DPH.

Homogeneity of LW Peptide Incorporation into Vesicles. One factor that might contribute to the difference between thermal transitions detected by DPH and LW peptides is inhomogeneous incorporation of the LW peptide into vesicles.

[2] The increase in quenching at a low temperature in the presence of cholesterol in Figure 6A may actually be due to a loss of domain formation as the temperature decreases. This would be expected if a mixture of lipids are miscible not only when they are all in the disordered state at high temperature but also when they are all in the ordered state at low temperature. Under such conditions, strong quenching should be observed both in bilayers that are the fully ordered state and the fully disordered fluid.

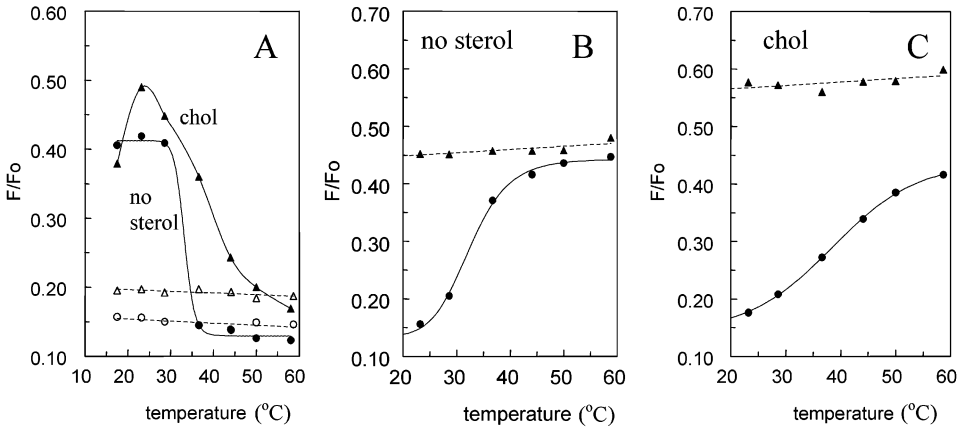

FIGURE 6: Quenching of DPH and LW peptide fluorescence in lipid mixtures with high DPPC content. (A) Quencher-containing vesicles composed of 0.25 mol % DPH and (closed triangles) 3:1:1.33 DPPC/12SLPC/cholesterol; (open triangles) 3:1:1.33 DOPC/12SLPC/cholesterol; (closed circles) 3:1 DPPC/12SLPC; or (open circles) 3:1 DOPC/12SLPC. (B) Vesicles composed of 2 mol % LW peptide and (circles) 3:1 DPPC/12SLPC or (triangles) 3:1 DOPC/12SLPC. (C) Vesicles composed of 2 mol % LW peptide and (circles) 3:1:1.33 DPPC/12SLPC/cholesterol or (triangles) 3:1:1.33 DOPC/12SLPC/cholesterol. Other experimental procedures and conditions are as in Figures 1 and 2.

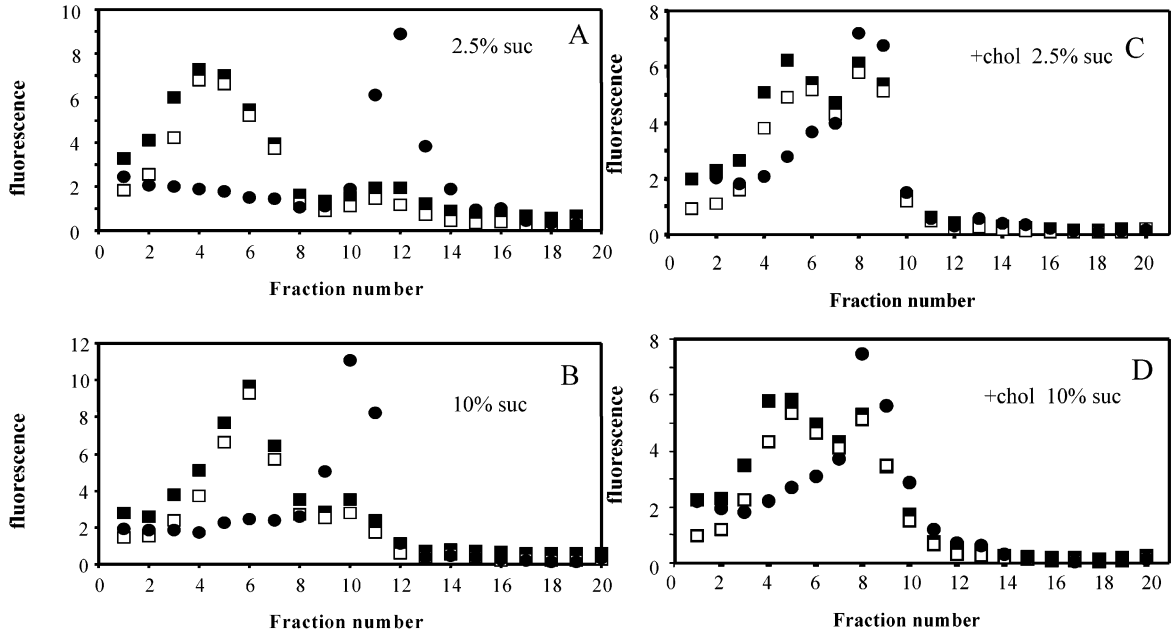

FIGURE 7: Sucrose gradient analysis of peptide-containing vesicles. Peptide-containing samples contained ethanol dilution vesicles composed of 5 μM LW peptide and 100 μM of (A and B) 3:1 DPPC/DOPC with a lipid marker of 0.25 mol % rhodamine-PE or (C and D) 3:1:1.33 DPPC/DOPC/cholesterol with 0.25% rhodamine-PE. Peptide-containing vesicles were mixed 1:1 (v/v) with an equal amount of peptide-lacking vesicles that were otherwise identical except that they contained 0.5 mol % NBD-PE as a lipid marker in place of rhodamine-PE. (A and C) Samples prepared in 2.5% (w/v) sucrose and (B and D) samples prepared in 10% (w/v) sucrose. Symbols: (circles) fluorescence of NBD-PE in vesicles lacking LW peptide; (open squares) fluorescence of rhodamine-PE in vesicles containing LW peptide; and (closed squares) Trp fluorescence of the LW peptide. Gradient bottom is at left on the *x*-axis.

If LW peptide is incorporated into a small subset of vesicles, their very high peptide-to-lipid ratio might disturb lipid phase melting behavior. Therefore, vesicle inhomogeneity was examined by sucrose gradient centrifugation. The samples analyzed contained a mixture of two vesicle preparations. Each preparation was composed of 3:1 DPPC/DOPC, but one contained LW peptide (plus rhodamine-PE as a marker), while the second lacked peptide (and contained NBD-PE as a marker). Figure 7 shows that, regardless of whether cholesterol was present or absent, the peptide-to-rhodamine fluorescence ratio was only weakly dependent on the position in the sucrose gradients, increasing only by roughly 2-fold in the densest fractions. These results suggest that the LW peptide was distributed throughout the population of rhodamine-containing vesicles.

These results did not entirely eliminate the possibility of inhomogeneous peptide incorporation because of possible vesicle size artifacts. Large vesicles contain more trapped solution than small vesicles, and the trapped solution can have a different density than the lipid bilayer and the external solution. It is conceivable that an inhomogeneous sample could form in which smaller vesicles lacking peptide could have the same density as larger vesicles containing peptide and thus appear in the same fractions. This would require that in the peptide-containing preparation there would be a subpopulation of protein-free vesicles that were significantly smaller than the vesicles containing peptide. This seems improbable, but to firmly eliminate the possibility of such artifacts, vesicles were loaded with sucrose at a low (Figure 7A,C) or high (Figure 7B,D) concentration to alter the density

of their internal solutions. Comparison of the fractionation profiles shows that internal sucrose concentration had only a minor effect, demonstrating that the influence of the internal solution was negligible. Similar results were obtained with control DOPC samples, with or without cholesterol (not shown). We conclude that inhomogeneous peptide insertion does not explain the differences between LW peptide and DPH thermal profiles. Other factors that might contribute to the difference between DPH and LW peptide behavior, including the possibility of the presence of residual domains at high temperature, are considered in the Discussion.

It should be noted that although most vesicles containing LW peptide (rhodamine-labeled population) appeared at a higher density than the NBD-labeled vesicles lacking peptide, a significant percent of the rhodamine-labeled vesicles appeared in the peak of NBD-labeled vesicles and vice versa. We speculate that this resulted from some degree of aggregation or fusion between the NBD- and the rhodamine-labeled vesicles.

DISCUSSION

Exclusion of Transmembrane Proteins from Ordered Domains. In this study, a fluorescence quenching was used to show that a membrane-inserted peptide with a hydrophobic polyLeu core was relatively excluded from both gel and liquid-ordered lipid domains. The depletion of integral membrane proteins and polypeptides from gel-phase domains has long been recognized (*21*, *23*, *38*, *58*). The basis of this exclusion is likely to be the inability of TM polypeptides to pack tightly with the closely packed lipids of the gel phase. Because liquid-ordered domains also are characterized by tight lipid packing (*9*, *12*), a similar phenomenon probably explains the exclusion of TM polypeptides from liquid-ordered domains. The observation that the exclusion of the LW peptide from ordered domains was similar in the presence of 0, 25, or 33 mol % cholesterol indicates that cholesterol concentration is not a critical factor in exclusion. In addition, the observation of exclusion from ordered domains containing sphingomyelin in place of DPPC, or ergosterol in place of cholesterol, indicates that exclusion is not strongly dependent on the exact structure of the lipids participating in ordered-domain formation. The observation that a peptide with a less hydrophobic alternating LeuAla sequence is also excluded from ordered domains as judged by detergent solubility (*20*) suggests that exclusion from ordered domains is not restricted to polyLeu sequences.

Factors Influencing Quenching: Quantum Yield, Complex Lipid Mixtures, and Domain Size and Shape. Previous studies have shown that the quenching of a fluorescent molecule in binary lipid mixtures containing multiple phases is dependent upon, and can be used to determine, K_p for a fluorescent molecule (*21*, *50*, *59*−*61*). A number of factors complicate K_p calculations. One complication is a difference in quantum yield for a fluorophore in ordered and disordered domains in the absence of a quencher. In such cases, F/F_o values will be weighted toward the value in the domain in which the quantum yield in the absence of quencher is higher. This factor could be disregarded in this study (see Appendix). Another complication is the use of ternary lipid mixtures, which have more complex phase behavior than binary mixtures (*18*). Methods for the determination of relative K_p values have been developed for such systems (*18*, *62*) but are most useful when comparing a series of molecules carrying identical fluorescent labels.

A third complication is domain size. Raft size and its control in vivo are among the more controversial aspects of raft behavior. Raft size is of particular importance because it may control raft function. In cells, it appears that rafts are usually submicroscopic in size but may become large under some conditions (*3*, *8*, *45*, *63*, *64*). Lipid domains in the model membrane can also vary between microscopic and submicroscopic depending on experimental conditions (*44*). To address the effects of domain size in our experiments, we developed a theoretical analysis of the relationship between quenching and domain size. The analysis revealed that quenching can be significantly altered when domains are small enough that a significant fraction of the molecules in a domain reside at the domain edge. It also showed that when domain size is extremely small, quenching can be similar to that in homogeneous bilayers. The reason for this is that a molecule at a domain boundary usually has a lipid environment that is closer to the overall average lipid composition in the sample than a molecule in the core of a domain. Nevertheless, our analysis suggests that domains as small as 30 molecules should still be detected by nitroxide-induced fluorescence quenching (see Appendix). It is noteworthy that the sensitivity of quenching to domain size might allow the estimation of domain size in a range that is not easily accessible to methods such as light microscopy. At present, atomic force microscopy in supported bilayer systems is probably the only method with an equivalent or higher level of resolution (*65*−*68*).

It should be cautioned that the model used to analyze the affect of domain size upon quenching was limited in several ways. We did not attempt to calculate precisely how quenching values would be affected by domain shape, by conditions in which the domain size has a complex dependence on lipid concentration, or by conditions in which domains of the same type come very close to each other. In general terms, domain shapes with a higher edge-to-core ratio would tend to show edge effects more strongly. Large circular domains would be influenced the least by edge effects. Very small submicroscopic domains are likely to be irregular in shape, which would tend to increase the edge-to-core ratio. Differences in quantum yield in different domains would also affect the sensitivity of quenching to domain size (see Appendix). The effect of such variables will be interesting to analyze in more detail if experimental systems with an extremely small domain size are identified.

Effect of the Range of Quenching Interactions upon Sensitivity to Domain Size. Although in this report the effects of domain size were analyzed for short-range nitroxide-induced quenching, domain size would also be an issue in the interpretation of other submicroscopic fluorescence phenomena, such as dipole−dipole energy transfer. The longer the spectroscopic interaction range, the larger the amount of a domain that is at the effective domain edge, and the greater the influence of domain size upon spectroscopic behavior. Thus, the effects of small domain size should be much less of a problem when short-range quenchers, such as nitroxide or brominated lipids, are used than it is when energy transfer pairs with large (30−50 Å) interaction distances (R_o) are used. This factor may reconcile the

different results reported for phase separation in mixtures of DPPC and dilauroylphosphatidylcholine (DLPC) in the presence of cholesterol. Using a brominated DLPC analogue that should be similar to DLPC in physical properties, Silvius et al. detected the formation of separate DPPC-rich domains in DPPC/DLPC mixtures that contained as little as 5% DPPC, when 15 mol % cholesterol was also present (*69*). Using energy transfer probes under similar experimental conditions, Feigenson and Bubholtz only detected DPPC-rich domain formation at >60% DPPC (*44*). In the future, a side-by-side comparison of raft formation by short-range quenchers and energy transfer has the potential to allow the estimation of the size of submicroscopic rafts.

Factors Influencing Quenching: Association of Molecules with Raft Edges. We have also shown that fluorescent molecules specifically associating with domain edges would exhibit unique quenching behavior. Quenching would not only reflect K_p but also the association constant with the domain boundary, which we named K_{edge}. Although experimental results indicate that LW peptide does not concentrate near domain edges, it may be possible to use quenching to identify domain edge-seeking molecules in the future.

The determination of whether molecules accumulate at raft edges is of particular interest because of the potential biological significance of raft boundaries for membrane structure and function. Mitchell and Litman have proposed that certain lipids with one saturated chain and one highly unsaturated chain orient such that their saturated chains cluster together with cholesterol (*70*). Since many natural phospholipids tend to have one saturated and one unsaturated chain, it is conceivable that some types may tend to concentrate at the edges of ordered domains with a sphingolipid/cholesterol core in an analogous manner. Such edge-preferring lipids could have a large impact upon raft structure and function because they would promote the formation of domain boundaries. With a fixed amount of raft-forming lipids, the introduction of edge-preferring lipids would tend to increase the domain perimeter-to-area ratio, requiring a decrease in domain size linked to an increase in domain number or a decrease in the smoothness of raft edges (*8*).

Some proteins may also accumulate at domain edges. Interactions between a protein with a strong affinity for rafts and one that is excluded from rafts might cause them to accumulate at domain boundaries. In addition, TM proteins covalently modified by saturated acyl chains, or bound to raft-associated lipids, might orient such that the TM polypeptide remained outside a raft while the palmitate chains or bound lipid were immersed within a raft. Such edge localization could be functionally important. It could increase local protein concentration in one dimension as well as control the interaction of membrane components with each other.

Difference between Temperature Dependence of LW and DPH Quenching. As noted in the Results, the thermal transitions detected by changes in the fluorescence of the LW molecules tended to be broader than those detected by DPH fluorescence. We showed that this was unlikely to result from inhomogeneous incorporation of peptide into a subset of vesicles. Instead, this difference may stem in part from the different partitioning behavior of the two probes. Theoretical analysis showed that the thermal dependence of the quenching of molecules partitioning strongly into fluid domains, like the LW peptide, has a slightly more gradual temperature dependence than that of molecules, which like DPH, partition equally between fluid and ordered domains (calculations not shown).

This difference might also reflect a local perturbation of lipid phase behavior induced by LW peptide. In this regard, it is noteworthy that at high temperature, LW quenching in samples containing DPPC plus cholesterol or ergosterol seemed to indicate the presence of residual domains as judged by stronger quenching than in control samples. This difference in quenching was not noticeable for DPH fluorescence. One possible explanation for this is that LW molecules locally altered the mixing behavior of lipids such that small 12SLPC-enriched lipid domains locally persisted specifically around the peptide molecules. On the other hand, it is possible that the LW peptide was incorporated into a subset of vesicles enriched in 12SLPC. The observation that the strong quenching of peptide fluorescence was largely abolished at high temperatures in samples containing DPPC ruled out the possibility that LW peptide was incorporated into a subset of vesicles greatly enriched in 12SLPC relative to the average sample composition. However, incorporation of LW peptide into vesicles slightly enriched in 12SLPC could not be ruled out.

Effect of Strong Partition into Fluid Domains on Domain Formation in Natural Membranes. The presence of TM proteins strongly excluded from ordered domains could significantly increase the formation of disordered domains in cell membranes. We saw possible evidence for such effects in bilayers with very high levels of saturated lipid (i.e., under conditions in which all, or almost all, of the bilayer would form ordered domains in the absence of peptide). The exact balance between ordered and disordered domains in vivo is unclear, but it has been proposed that a large fraction of the plasma membrane may consist of ordered domains (*71*−*73*). Therefore, it is possible that the high content of TM proteins in natural membranes influences lipid domain formation. Very recently, Ge et al. have suggested this as an explanation for electron spin resonance data obtained in mast cell membranes (*74*).

APPENDIX: RELATIONSHIP BETWEEN DOMAIN SIZE AND QUENCHING

To determine the effect of the domain size upon quenching, it is necessary to account for both the number of fluorophores in each domain and the number of fluorophores at the domain edges. The fraction of the fluorescent molecules in ordered and disordered domains is related to the total fraction of the bilayer that is in the ordered and disordered states and to the value of K_p for the fluorescent molecules. K_p is defined as the ratio of concentrations of the fluorescent molecule, which we name molecule M, in the disordered domains [M_{dd}] to that in ordered domains [M_{od}]. If the fraction of the bilayer that is in the disordered state is α, then that in the ordered state is $1 - \alpha$ and

$$K_p = [M_{dd}]/[M_{od}] = (\text{mol of } M_{dd}/\text{mol of } M_{od})(A_{od}/A_{dd}) = (\text{mol of } M_{dd}/\text{mol of } M_{od})((1 - \alpha)/\alpha) \quad (1)$$

where A_{dd} and A_{od} are the total areas of membrane that are in the form of disordered or ordered domains, respectively.

(For simplicity, we consider cases in which the quencher only affects fluorophores in the same leaflet of the bilayer. Under these conditions, the bilayer is essentially a two-dimensional object, so that area is the analogue of volume in a three-dimensional solution.) Rearrangement of eq 1 allows the calculation of the fraction of fluorescent molecules located in ordered and disordered domains, $f\mathrm{M}_{od}$ and $f\mathrm{M}_{dd}$, respectively.

$$f\mathrm{M}_{od} = \text{mol of } \mathrm{M}_{od}/(\text{mol of } \mathrm{M}_{od} + \text{mol of } \mathrm{M}_{dd}) = (1 - \alpha)/(1 - \alpha + \alpha K_p) \quad (2)$$

$$f\mathrm{M}_{dd} = \text{mol of } \mathrm{M}_{dd}/(\text{mol of } \mathrm{M}_{od} + \text{mol of } \mathrm{M}_{dd}) = \alpha K_p/(1 - \alpha + \alpha K_p) \quad (3)$$

Two regions within a domain must be distinguished: the domain edge and the domain core. Considering the case in which a number of ordered domains exist and are surrounded by a single continuous disordered lipid domain, and in which molecule M has the same concentration in the core and at the edge of a domain, the fraction of fluorescent molecules within, but at the edges of, ordered domains ($f\mathrm{M}_{ode}$) is

$$f\mathrm{M}_{ode} = f\mathrm{M}_{od}(A_{ode}/A_{od}) = ((1 - \alpha)/(1 - \alpha + \alpha K_p))(A_{ode}/A_{od}) \quad (4)$$

where A_{ode}/A_{od} is the ratio of the total area at the edge of ordered domains (A_{ode}) to the total area of the ordered domains. The fraction of molecules in ordered domains, but not near their edge (i.e., in the core of ordered domains (fM_{odc})), is then given by

$$f\mathrm{M}_{odc} = f\mathrm{M}_{od} - f\mathrm{M}_{ode} = f\mathrm{M}_{od}(1 - A_{ode}/A_{od}) = ((1 - \alpha)/(1 - \alpha + \alpha K_p))(1 - A_{ode}/A_{od}) \quad (5)$$

Since the ratio of the total area in ordered domains to the total area in the disordered domains (A_{od}/A_{dd}) equals $(1 - \alpha)/\alpha$, the fraction of molecules within disordered domains but at the edge of an ordered domain ($f\mathrm{M}_{dde}$) is then given by

$$f\mathrm{M}_{dde} = f\mathrm{M}_{dd}(A_{dde}/A_{dd}) = f\mathrm{M}_{dd}(A_{dde}/A_{od})((1 - \alpha)/\alpha) = (\alpha K_p/(1 - \alpha + \alpha K_p))(A_{dde}/A_{od})((1 - \alpha)/\alpha) \quad (6)$$

where A_{dde} is the total area at the edge of the disordered domains.

Finally, the fraction of molecules in the disordered regions of the membranes that are not near the edge of an ordered domain (i.e., the fraction of molecules in the core of the disordered domain ($f\mathrm{M}_{ddc}$)) is

$$f\mathrm{M}_{ddc} = f\mathrm{M}_{dd} - f\mathrm{M}_{dde} = (\alpha K_p/(1 - \alpha + \alpha K_p))(1 - [(A_{dde}/A_{od})((1 - \alpha)/\alpha)]) \quad (7)$$

To make use of these equations, the fraction of a domain that is in its core and at the edge must be defined. The relative dimensions of the edge and core of a domain are related to the size of the domain and the range of the quenching interaction. For domains with the shape of long stripes of width w and a quenching interaction of range x (see Figure 9), the general relationship between domain size and edge size can be stated as

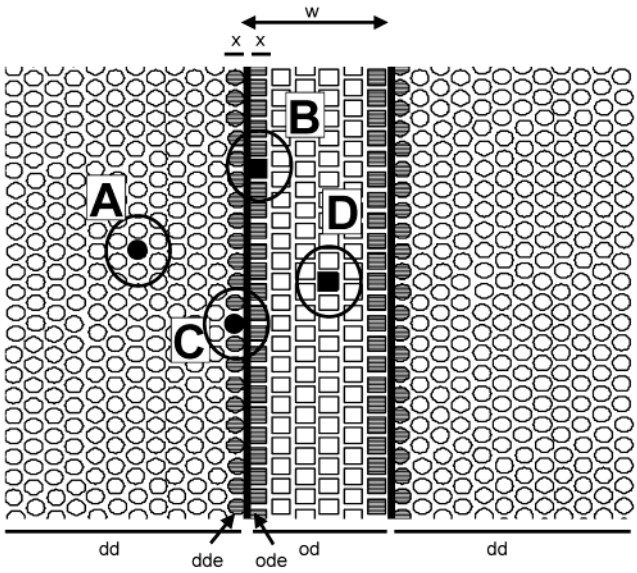

FIGURE 8: Schematic illustration of bilayers containing stripe-shaped domains. Lipids in disordered domains are shown as circles. Lipids in ordered domains are shown as squares. Lipids at domain edges are shown shaded. Labels: x = width of domain edge, w = width of domain, dd = disordered domain, od = ordered domain, dde = edge of disordered domain, and ode = edge of ordered domain. The local environment of four molecules (A−D) is also shown. Notice that molecule A in the core of the disordered domain is surrounded by six molecules in the disordered domain, and molecule B at the edge of the ordered domain is surrounded by two molecules in disordered domains and four molecules in ordered domains, etc.

$$A_{ode}/A_{od} = 2x/w \quad (8)$$

$$A_{dde}/A_{od} = 2x/w \quad (9)$$

Notice that $A_{ode} = A_{dde}$ since the edge of the ordered and disordered domains have the same width (Figure 8). It is possible to define x and w in terms of lipid diameters (i.e., n lipid molecules thick or angstroms). Since natural phospholipids have a cross-sectional area close to 50−70 Å^2 depending on the lipid state, the diameter of one lipid molecule is equivalent to 7−9 Å.

To define the dimensions of x for the experiments in this paper, we must consider the case of quenching by nitroxide-labeled lipids. Nitroxide quenching is short-range, and roughly speaking only nitroxides attached to the nearest lipid neighbors of a fluorescent molecule should be close enough to quench fluorescence (*54*−*56*). Therefore, only fluorophores in the layer of the lipid at the immediate domain edge can be quenched by nitroxide-labeled lipids that are outside the domain in which they are located. In other words, the domain edge (x) is one lipid layer wide (see Figure 8).

To estimate the effect of domain size upon fluorescence quenching, the quenching of molecules within the core and at the edges of both ordered and disordered domains must also be calculated. We consider a mixture of a nitroxide-labeled lipid and an unlabeled lipid and use a model in which the lipids in the bilayer exhibit typical close lateral packing, such that each lipid is surrounded by six others (Figure 8). The quenching of lipid-sized fluorescent molecules by

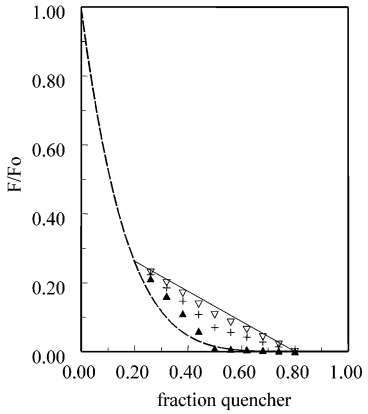

FIGURE 9: Calculated dependence of quenching of a DPH-like probe ($K_p = 1$) in bilayers composed of mixtures of DPPC and 12SLPC as a function of domain size. The fraction quencher is the fraction of phospholipid molecules that are 12SLPC. The dashed line shows quenching in a homogeneous bilayer that does not form domains. Quenching in the region of phase coexistence is calculated for the case in which ordered domains contain 20 mol % 12SLPC and disordered domains contain 80 mol % 12SLPC. It is assumed that the least abundant components (disordered domains or ordered domains) exist as small domains surrounded by a continuous domain formed by the more abundant component (i.e., for [fraction quencher] < 0.5, disordered domains are small, and for [fraction quencher] > 0.5, ordered domains are small). Quenching is shown in the two-phase (domain-forming) range of compositions in which domains (solid line) are of infinite size (i.e., with no molecules near domain edge); (open triangles) have 20% of molecules near the domain edge ($2x/w = 0.2$); (crosses) have 50% of molecules near the domain edge ($2x/w = 0.5$); or (closed triangles) have 100% of molecules near the domain edge ($2x/w = 1$).

nitroxide-labeled lipids can be then approximated by the expression $F/F_o = (1 - C)^6$ where C is the local concentration of the quencher lipid in mol fraction units (*22*, *54*). This expression is valid for a fluorescent molecule in the core of a domain (see Figure 8, molecules A and D) if it is fully quenched when any of its six neighbors carries a nitroxides (*22*, *54*). At the edge of a domain, F/F_o depends on how many neighbors are within the domain, how many are outside the domain, and the value of C within and outside the domain. For domains with straight boundaries (e.g., a long rectangular stripe), four lipids will be within the same domain as the fluorophore, and two will be outside the domain (see Figure 8, molecules B and C). Thus, at the domain edge, $F/F_o = (1 - C_{\text{inside domain}})^4(1 - C_{\text{outside domain}})^2$.

Combining the previous equations allows the calculation of the dependence of quenching upon domain size. For simplicity, we assume that the fluorescent molecule in ordered and disordered domains has the same quantum yield when in the absence of a quencher. The general expression for F/F_o in a sample is then

$$F/F_o = \sum (\text{fraction of fluorescent molecules in an environment})(F/F_o \text{ in that environment}) = \sum fM_i(F/F_o)_i \quad (10)$$

Where i represents each environment, and the sum is over all environments. This can be expanded into four terms

$$F/F_o = fM_{odc}(F/F_o)_{odc} + fM_{ode}(F/F_o)_{ode} + fM_{ddc}(F/F_o)_{ddc} + fM_{dde}(F/F_o)_{dde} \quad (11)$$

Where $F/F_{o\ odc}$ stands for F/F_o in the core of the ordered domains, etc. For long rectangular domains, substituting eqs 4−9 and the previous expressions describing the dependence of F/F_o upon C into eq 11 gives

$$F/F_o = [((1-\alpha)/(1-\alpha+\alpha K_p))(1-(2x/w))] \times (1-C_o)^6 + [((1-\alpha)/(1-\alpha+\alpha K_p))(2x/w)] \times (1-C_o)^4(1-C_d)^2 + [(\alpha K_p/(1-\alpha+\alpha K_p)) \times (1-(2x/w)((1-\alpha)/\alpha))](1-C_d)^6 + [((1-\alpha)K_p/(1-\alpha+\alpha K_p))(2x/w)](1-C_d)^4(1-C_o)^2 \quad (12)$$

where C_o is the concentration of the quencher in the ordered domains (as a mol fraction of total lipid in the domain), and C_d is the concentration of the quencher in the disordered domains. Notice that this equation is not valid when $2x > w$. If $2x > w$, the area at the edge of a domain would exceed the total area within that domain, which is physically impossible.

Analogous equations can be developed for the case in which a bilayer contains disordered domains surrounded by a continuous ordered domain. In this case, w is the width of the disordered domain. The fraction of fluorescent molecules within, but at the edge of the disordered domains, is given by

$$fM_{dde} = fM_{dd}(A_{dde}/A_{dd}) = (\alpha K_p/(1-\alpha+\alpha K_p))(A_{dde}/A_{dd}) \quad (13)$$

The fraction of molecules in the core of the disordered domains is given by

$$fM_{ddc} = fM_{dd} - fM_{dde} = fM_{dd}(1 - A_{dde}/A_{dd}) = ((\alpha K_p)/(1-\alpha+\alpha K_p))(1 - A_{dde}/A_{dd}) \quad (14)$$

The fraction of molecules in the ordered domains, but at the edge of a disordered domain, is given by

$$fM_{ode} = fM_{od}(A_{ode}/A_{od}) = fM_{od}(A_{ode}/A_{dd})(\alpha/(1-\alpha)) = ((1-\alpha)/(1-\alpha+\alpha K_p))(A_{ode}/A_{dd})(\alpha/(1-\alpha)) \quad (15)$$

Finally, the fraction of molecules in the core of the ordered regions is given by

$$fM_{odc} = fM_{od} - fM_{ode} = ((1-\alpha)/(1-\alpha+\alpha K_p)) - ((1-\alpha)/(1-\alpha+\alpha K_p))(A_{ode}/A_{dd})(\alpha/(1-\alpha)) = ((1-\alpha)/(1-\alpha+\alpha K_p))(1-(A_{ode}/A_{dd})(\alpha/(1-\alpha))) \quad (16)$$

Substitution of these expressions into eq 11 gives

$$F/F_o = [((1-\alpha)/(1-\alpha+\alpha K_p))(1-(\alpha/(1-\alpha)) \times (2x/w))](1-C_o)^6 + [(\alpha/(1-\alpha+\alpha K_p))(2x/w)] \times (1-C_o)^4(1-C_d)^2 + [((\alpha K_p)/(1-\alpha+\alpha K_p)) \times (1-2x/w)](1-C_d)^6 + [(\alpha K_p/(1-\alpha+\alpha K_p))(2x/w)] \times (1-C_o)^2(1-C_d)^4 \quad (17)$$

Figure 9 shows the effect of domain size on the fluorescence quenching of a molecule that (like DPH) has a K_p close to 1. Notice that edge effects become significant when $2x/w$

is greater than 0.2 (i.e., for domains that are 10 molecules wide) and that edge effects distort quenching strongly when $2x/w$ is as large as 0.5−1 (crosses and filled triangles). In fact, when domains are very small (filled triangles), the quenching behavior approaches that in homogeneous bilayers. Also notice that at a quencher mol fraction of 0.5, the small domain size can result in anomalous quenching, such that there is increased quenching relative to that in homogeneous bilayers under conditions in which the presence of large domains decreases the quenching relative to that in homogneous bilayers.

The quenching equations derived previously must be modified for application to the Trp fluorescence of transmembrane polypeptides or proteins. As a consequence of their larger cross-sectional area relative to lipids, a lipid-exposed Trp residue in a polypeptide will have fewer lipid neighbors than a lipid-sized molecule. Previous estimates give a crude value of 1−2 lipid neighbors per Trp for membrane proteins (*54*), and the data for the LW peptide used in this report also fits a value close to 2 (calculation not shown). Thus, for the Trp fluorescence of LW peptide in the core of a domain, $F/F_o = (1 - C)^2$. At the edge of a domain, where half of the lipid neighboring a Trp would be inside the domain and half outside, one can estimate that $F/F_o = (1 - C_{\text{inside domain}})(1 - C_{\text{outside domain}})$. In this case, eqs 12 and 17 become

$$F/F_o = [((1 - \alpha)/(1 - \alpha + \alpha K_p))(1 - (2x/w))] \times (1 - C_o)^2 + [(((1 - \alpha)(1 + K_p))/(1 - \alpha + \alpha K_p)) \times (2x/w)](1 - C_o)(1 - C_d) + [(\alpha K_p/(1 - \alpha + \alpha K_p)) \times (1 - (2x/w)((1 - \alpha)/\alpha))](1 - C_d)^2 \quad (18)$$

and

$$F/F_o = [((1 - \alpha)/(1 - \alpha + \alpha K_p))((1 - (\alpha/(1 - \alpha))) \times (2x/w))](1 - C_o)^2 + [(\alpha(1 + K_p)/(1 - \alpha + \alpha K_p)) \times (2x/w)](1 - C_o)(1 - C_d) + [(\alpha K_p/(1 - \alpha + \alpha K_p)) \times (1 - 2x/w)](1 - C_d)^2 \quad (19)$$

respectively.

The previous analysis only considered the quenching of molecules whose behavior can be fully defined by their partition between ordered and disordered domains. A different fluorescence quenching pattern would be observed for a molecule is located at the boundaries between ordered and disordered domains. When domains are present, F/F_o for a polypeptide that sits exclusively at the domain edges would approximately be given by

$$F/F_o = (1 - C_o)(1 - C_d) \quad (20)$$

since in binary lipid mixtures, C_o and C_d are invariant when ordered and disordered domains coexist, and F/F_o would be independent of the quencher concentration for these compositions.

The analysis of the dependence of the quenching upon domain size can also be extended to circular domains. For circular ordered domains of radius r that are embedded in a continuous disordered phase, the following equations apply:

$$A_{ode}/A_{od} = (\pi r^2 - \pi(r - x)^2)/\pi r^2 = 1 - (1 - x/r)^2 \quad (21)$$

$$A_{dde}/A_{od} = (\pi(r + x)^2 - \pi r^2)/\pi r^2 = (1 + x/r)^2 - 1 \quad (22)$$

The exact solution for the dependence of the quenching on the domain radius is algebraically complex for circular domains in a hexagonally packed lipid lattice. However, it is possible to crudely estimate how many lipid molecules must be in a circular domain to allow the domain to be easily detected by nitroxide lipid-induced quenching. On the basis of the preceding analysis, it can be estimated that to easily detect ordered domains surrounded by a continuous disordered domain, $A_{ode}/A_{od} < 0.5$, for a probe having $K_p = 1$. Solving eq 21 with $A_{ode}/A_{od} = 0.5$ gives $x/r = 0.293$. If x is approximately 8 Å (see previously), then r is about 25−30 Å. This means that the area of a domain (πr^2) must be about 2000 Å^2 for easy detection. Since lipid molecules occupy about 70 Å^2, this is equivalent to a domain of about 30 lipid molecules, showing that nitroxide quenching should be able to detect very small domains. Similarly, if edge effects are negligible when $A_{ode}/A_{od} < 0.2$, then nitroxide quenching will not be significantly influenced by edges when $r > 75$ Å (i.e., for domains of over 250 lipid molecules).

It should be noted that the analysis described in eqs 10−22 ignores corrections needed when the quantum yield of a fluorophore is significantly different in the ordered and disordered state. The corrected form of eq 10 would be

$$F/F_o = \sum(f\mathrm{M}_i(F/F_o)_i N_i)/\sum(f\mathrm{M}_i N_i) \quad (10a)$$

where $N_i = F_{oi}/F_{o1}$, which is the ratio of F_o values in environment i to that in the first environment.

Use of the corrected form of eq 10 is unnecessary when the quantum yield difference is small or when the fluorescent molecules partition very strongly into one particular type of domain. Since F_o is proportional to the quantum yield, in practice a correction can be ignored when F_o is not dependent upon the fraction of the bilayer that is in the disordered state (α). Since F_o for the LW peptide was relatively constant over the entire range in which disordered and ordered domains coexist (i.e., 12SLPC concentrations between 0.2 and 0.8) (see Figure 4C), we did not attempt to calculate corrected K_p values for LW peptide. A second reason not to correct K_p was that the corrected previous equation assumes that the difference in quantum yield is not dependent upon the concentration of the fluorescence molecule in a particular domain. We suspect that the LW peptide aggregates within ordered bilayers and that this aggregation results in self-quenching that decreases Trp quantum yield. (Self-quenching of Trp at high local concentration has been observed previously (*53*).) If this is correct, and LW peptide concentration within ordered domains is greatly decreased in the presence of disordered domains (due to its partition out of the ordered domains), then the quantum yield correction would exaggerate how much of the LW peptide was in the ordered domains.

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