Quenching of Tryptophan Fluorescence by Brominated Phospholipid†

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ABSTRACT: Bromolipids [1-palmitoyl-2-(dibromostearoyl)phosphatidylcholine] with bromines at the 4,5-, 6,7-, 9,10-, 11,12-, and 15,16-positions were used to examine the fluorescence quenching of a synthetic, membrane-spanning peptide (Lys_2-Gly-Leu_8-Trp-Leu_8-Lys-Ala-amide) incorporated into both small and large unilamellar vesicles. The peptide–lipid vesicles were analyzed to show that at least 75% of the peptide was in a transbilayer configuration, placing the single tryptophan in its predicted place in the center of the bilayer. Quenching profiles of the peptide in bromolipid showed maximal (90%) quenching by the 15,16-bromolipid, indicating that the bromolipids can accurately locate the position of a tryptophan in the bilayer. The quenching by the other bromolipids decreased with an r^6 dependence and an apparent R_0 of 9 Å. In addition, indole in methanolic solution was subjected to quenching by a variety of mono- and dibrominated hydrocarbons. The quenching was analyzed, by using a modified Stern–Volmer equation, and found to be greatly dependent upon the number and positioning of the bromines. Monobromobutanes were found to have a quenching efficiency of only 7% while dibromobutanes, with bromines on adjacent carbon atoms, had efficiencies of over 80%. In addition, the dibromobutanes exhibited significant "static" quenching whereas the monobrominated butanes did not. These data suggest that the bromolipids are more appropriately defined as short-range quenchers rather than strictly contact quenchers.

The quenching of tryptophan fluorescence by various membrane-bound probes has been used extensively to determine the depth of proteins in the membrane. Initial estimations of protein depth relied on dipole–dipole (Forster) energy transfer from the tryptophan(s) of the protein to an appropriate acceptor molecule located on a relatively distant probe (Koppel et al., 1979; Kleinfeld, 1985). More recently, spin-labeled probes (Chattopadhyay & London, 1987; Voges et al., 1987) and brominated phospholipids (East & Lee, 1982; Jain & Maliwal, 1985; Markello et al., 1985) have been developed for use in the determination of depth. Whereas energy-transfer probes can quench over large distances, spin-labeled probes and brominated lipids are thought to be collisional quenchers and therefore must come in close contact for quenching to occur. The definition of close contact is, however, somewhat obscure. For some systems, it appears that overlapping van der Waals radii are required for quenching. In other systems, close contact is defined as the close approach of the fluorophore–quencher pair to within 3–5 Å of each other. In the membrane, the definition of close contact becomes crucial as small distances become significant. It is important, therefore, to know the distance dependence of the system being studied.

In this laboratory, a series of brominated phosphatidylcholines were synthesized for use as depth-dependent probes. Bromines were placed on the 4,5-, 6,7-, 9,10-, 11,12-, or the 15,16- carbon atoms of the *sn*-2 acyl chain, thus providing a molecular ruler in the membrane. Markello et al. (1985) made use of the bromolipids[1] to determine the depth of the fluorescent tryptophans of the membrane-binding domain of cytochrome b_5 in the membrane. Their results, however, were inconsistent with those reported by Fleming et al. (1979) and Kleinfeld and Lukacovic (1985) using energy transfer. Thus, the ability of the bromolipids to accurately determine the depth of a protein in the membrane was questioned. This study was therefore undertaken in order to test the accuracy of the bromolipids as depth-dependent probes. A synthetic peptide containing a single tryptophan was obtained in order to test the bromolipids. The sequence of the peptide used in this study is Lys_2-Gly-Leu_8-Trp-Leu_8-Lys-Ala-amide. When the peptide assumes a transbilayer configuration, the tryptophan should be found in the center of the bilayer. This is indeed the case as is shown in this study. In addition, the quenching of indole by a series of brominated hydrocarbons was examined in order to better characterize the quenching by bromine. To our knowledge, this is the first study to examine the quenching of indole by different mono- and dibrominated compounds with the exception of a single study by Berlman (1973). However, in the study by Berlman, only two monobrominated compounds were examined. This distinction is important as the results of our studies indicate that there is a significant difference in the quenching by mono- and dibrominated compounds. Our studies also suggest that quenching by the bromolipids does not require contact between the tryptophan and the bromines but rather appears to have a distance dependence like that seen with energy transfer, only of a much shorter range.

Materials and Methods

Materials. 2-Bromobutane was from Aldrich, Milwaukee, WI. All other mono- and dibrominated compounds were from Pfaltz and Bauer, Waterbury, CT. The synthesis of the various bromolipids used in this study has been described in detail previously (Markello et al., 1985; Tennyson & Holloway, 1986). Peptide was a generous gift from Dr. Jay Fox of the University of Virginia Sequencing Facility. Peptide as obtained was found to contain a fluorescent impurity and

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[1] Abbreviations: POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; 4,5-BRPC, 1-palmitoyl-2-(4,5-dibromostearoyl)phosphatidylcholine; 6,7-BRPC, 1-palmitoyl-2-(6,7-dibromostearoyl)phosphatidylcholine; 9,10-BRPC, 1-palmitoyl-2-(9,10-dibromostearoyl)phosphatidylcholine; 11,12-BRPC, 1-palmitoyl-2-(11,12-dibromostearoyl)phosphatidylcholine; 15,16-BRPC, 1-palmitoyl-2-(15,16-dibromostearoyl)phosphatidylcholine; Tris-acetate, tris(hydroxymethyl)aminomethane acetate; EDTA, ethylenediaminetetraacetic acid; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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therefore had to be purified by chromatography before use. Crude peptide was taken up in 4:1 ethanol–88% formic acid, and approximately 1 mL (4 mg/mL) of the peptide was loaded onto a 21 × 300 mm LH-20 Sephadex column equilibrated in the same solvent. The peptide was eluted by gravity flow at an approximate rate of 50 mL/h; 1-mL fractions were collected and analyzed by measuring the absorbance between 250 and 300 nm. The peptide was found to elute near the void volume of the column while the fluorescent impurity eluted near the end of the fractionation range of the column. Fractions containing the peptide were pooled, dried down under nitrogen, and then taken up in methanol for storage. Peptide fractions from several runs were combined, and the concentration of peptide was determined from the absorbance at 280 nm, using an extinction coefficient of 5.55 mM^{-1}.

Fluorescence Measurements. Steady-state fluorescence measurements were made with an SLM 4800 (SLM Instruments, Urbana, IL). All measurements were made by using an excitation wavelength of 280 nm and were performed at 25 °C. All fluorescence measurements (except those made with LUVs) were corrected for inner filter effects by using the standard relation $F_{corr} = F_{ob}$ antilog $[(OD_{ex} + OD_{em})/2]$. The fluorescence measurements made with LUVs were corrected for absorption and scattering effects by using the dilution technique of Eisinger and Flores (1985). These measurements, of the fluorescence intensity for the peptide–lipid vesicles, were then corrected for variations in peptide content. Measurements of fluorescence lifetimes were made on an SLM 4800 which has been modified (ISS Inc., Champaign, IL) to provide a continuous range of modulation frequencies from 1 to 200 MHz (Gratton & Limkeman, 1983). A 280-nm band-pass filter was used for selection of the excitation wavelength from the xenon lamp, in this case.

Preparation of Peptide–Lipid Vesicles. Peptide was incorporated into POPC or bromolipid by mixing the peptide in methanol with the desired lipid in chloroform. The molar ratio of peptide to lipid was 1 to 200. The mixture was evaporated and then taken back up in cyclohexane and lyophilized overnight. Small unilamellar vesicles (peptide–lipid SUVs) were formed by taking the mixture up in 10 mM Tris–acetate–1 mM EDTA (pH 8.0 at 20 °C) and sonicating to clarity under nitrogen. SUVs were separated from large liposomes by differential ultracentrifugation (Barenholz et al., 1977). Large unilamellar vesicles (peptide–lipid LUVs) were formed by using the extrusion method (Hope et al., 1985). The lyophilized peptide–lipid mixture was hydrated with Tris buffer and cycled 10 times through the Extruder (Lipex Biomembranes Inc., Vancouver, British Columbia, Canada), using a 0.1-μm polycarbonate filter. The mean diameters of the resulting LUVs as determined by quazi-elastic laser light-scattering ranged between 101 and 108 nm.

TNBS Labeling of Peptide–Lipid Vesicles. Peptide–lipid vesicles were labeled with TNBS by using the procedure described by Litman (1973) and modified for use with Triton X-100. Briefly, peptide–POPC vesicles in Tris buffer were diluted to a final volume of 300 μL with Tris buffer, and then 100 μL of either 0.8 M $NaHCO_3$ (outer surface labeling) or 1.6% Triton X-100 in 0.8 M $NaHCO_3$ (total amino group labeling) was added and the sample mixed. A 1.5% TNBS solution was added and the sample mixed and allowed to incubate in the dark at room temperature for 1 h. The samples were acidified with either 1.2% Triton X-100 in 1.5 N HCl (outer determination) or 0.4% Triton X-100 in 1.5 N HCl (total determination), and the absorbance at 410 nm was measured. A solution of tryptophan in Tris buffer was used to construct a standard curve.

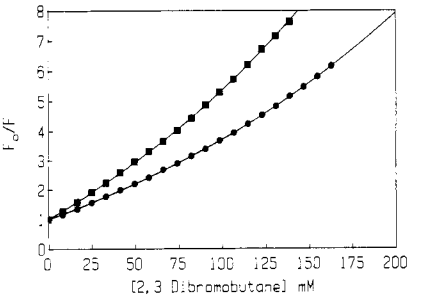

FIGURE 1: Fluorescence quenching of (■) indole or (●) peptide in methanol as a function of quencher (2,3-dibromobutane) concentration. F is the fluorescence intensity in the presence of quencher, and F_0 is the fluorescence intensity in the absence of quencher. F and F_0 were corrected for scattering and inner filter effects as described under Materials and Methods. Samples were 12.5 μM in indole or peptide. The solid line represents the fit to the equation $F_0/F = (1 + K_{SV}[Q])e^{V[Q]}$.

Results and Discussion

Fluorescence Quenching of Indole by Brominated Hydrocarbons. Collisional quenching in solution can be described by the classic Stern–Volmer relation:

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

Frequently, however, Stern–Volmer plots of quenching in solution are found to deviate from linearity [reviewed by Eftink and Ghiron (1981)]. Both positive and negative curvatures have been observed. While negative curvature has been explained by the existence of multiple fluorescing states or compound formation, positive curvature has been found to occur in a variety of systems which show no evidence of multiple excited states or molecular association (Keizer, 1983). To account for this upward curvature, a "static" quenching constant is invoked and the Stern–Volmer relation modified to contain an additional quenching term. Commonly, a "sphere of action" mechanism is used to describe the "static" quenching and the Stern–Volmer modified to contain the term $e^{V[Q]}$:

$$F_0/F = (1 + K_{SV}[Q])e^{V[Q]} \quad (2)$$

The "static" quenching constant V can be thought to represent a volume element around the fluorophore, for which the probability of quenching is unity if the quenching molecule is within this volume element at the moment of excitation. The "sphere of action" model has been used extensively in the past to explain the upward curvature which is observed with the quenching of indole and tryptophan by acrylamide (Eftink, 1983; Eftink & Jameson, 1982; Eftink & Ghiron, 1976).

In order to better understand the quenching of tryptophanyl fluorescence by bromolipid, a model system was designed to examine the quenching process in solution, thereby eliminating any complicating effects due to the membrane system. Indole and 2,3-dibromobutane were chosen as model compounds for studying the quenching in solution. 2,3-Dibromobutane was chosen as an analogue for the dibrominated acyl chain of the bromolipids, with the dibrominated butane chain being the smallest definable unit for the quenching reaction. Indole was chosen because its quenching in solution has been well characterized in numerous systems and it is known to have a single fluorescent lifetime, circumventing any complications due to multiple fluorescent states (Eftink & Hagaman, 1986). As can be seen in Figure 1, the quenching of indole by 2,3-dibromobutane exhibits significant upward curvature and therefore required the modified Stern–Volmer equation (eq 2) to describe the quenching. Table I gives the values of the

Table I: Stern–Volmer and Static Quenching Constants Determined for the Quenching of Indole by Various Brominated Hydrocarbons in Methanol[a]

quencher	K_{sv} (M^{-1})	V (M^{-1})
2,3-dibromobutane	31.9	2.48
1,2-dibromobutane	38.1	0.47
1,2-dibromocyclohexane	27.7	5.66
2,5-dibromohexane	10.3	0[b]
1,4-dibromobutane	8.71	0.65
2-bromobutane	2.57	0.02
1-bromobutane	2.28	0.12
acrylamide	25.9	1.87
2,3-dibromobutane[c]	11.3	2.45

[a] Fluorescence was monitored at the emission maximum. Data were fit to the equation $F_0/F = (1 + K_{sv}[Q])e^{V[Q]}$. F and F_0 were corrected for scattering and inner filter effects as described under Materials and Methods. All experiments were done at 25 °C with methanol as the solvent. [b] Value of V held fixed at 0 as data did not warrant use of the modified Stern–Volmer equation. [c] The solvent in this case contained 50% glycerol.

Stern–Volmer quenching constant K_{SV} and the "static" quenching constant V found for the quenching of indole in methanol by a series of different brominated hydrocarbons. In addition, the quenching of indole by acrylamide in methanol was measured and included for comparison to the brominated compounds. By comparing the K_{SV}'s for the different quenchers, it can be seen that the brominated compounds can be divided into three groups according to their K_{SV}'s. The first group contains the monobrominated compounds 1-bromobutane and 2-bromobutane. The K_{SV}'s of these two compounds are of the order of 2.5 M^{-1}. There was very little upward curvature seen with these compounds as is reflected by the negligible "static" quenching constant V. The second group contains the dibrominated compounds 1,4-dibromobutane and 2,5-dibromohexane. These compounds exhibit K_{SV}'s on the order of 10 M^{-1}. Again the "static" quenching is negligible. The third group contains the dibrominated compounds *trans*-1,2-dibromocyclohexane, 1,2-dibromobutane, and 2,3-dibromobutane. These compounds all have K_{SV}'s on the order of 30 M^{-1}. Both 2,3-dibromobutane and *trans*-1,2-dibromocyclohexane have significant "static" quenching constants. 1,2-Dibromobutane had the highest quenching rate, but had a negligible "static" quenching constant. Acrylamide, which is known to be an efficient quencher, exhibited K_{SV} and V values similar to 2,3-dibromobutane. Since the bimolecular quenching rate k_q for the quenching reaction can be determined from K_{SV} through τ_0 (the lifetime of the fluorophore in the absence of quencher), and since the efficiency of the quenching reaction is directly proportional to k_q, the value of the Stern–Volmer quenching constant reflects the efficiency of the quenching reaction (in this case). It can be concluded that the efficiency of the quenching reaction is increased (1) upon going from a monobrominated compound to a dibrominated compound and (2) as the bromines in the dibrominated compounds get closer together. The increase in K_{SV} is not simply attributable to the increase in the bromine content and suggests that coupling of the bromines may be occurring. The efficiency of the quenching reaction appears to be related to the degree of coupling for the bromines.

The actual efficiency of the quenching reaction can be calculated from the relation $\gamma = k_q/k_d$ where k_q is the observed quenching rate constant and is given by $k_q = K_{SV}/\tau_0$ and k_d is the diffusion-controlled rate constant and is given by $k_d = 4\pi N'(R_f + R_q)(D_f + D_q)$ where N' is $N/1000$. A value of 7 Å was used for the combined radii of the fluorophore–quencher pair. Diffusion coefficients for the molecules were calculated by using the Stokes–Einstein equation. These calculations result in an efficiency of 0.82 for the quenching of indole by 2,3-dibromobutane in methanol and an efficiency of 0.07 for the quenching of indole by 2-bromobutane in methanol. A value of 3.26 ns was measured for τ_0 (data not shown) and used in the above calculations.

As mentioned above, the "static" quenching constant, V, can be thought to represent a volume element around the fluorophore. The radius of this volume element, r (assuming a sphere), can be determined from V with the relation $V = 4\pi N'r^3/3$. For 2,3-dibromobutane, V equals 2.5 M^{-1}; hence, r, the radius of the quenching sphere, is 10 Å. This radius is larger than the sum of the molecular radii which is approximately 7 Å for the pair. This was found to be true for the quenching of peptide (Lys_2-Gly-Leu_8-Trp-Leu_8-Lys-Ala-amide) in solution as well. The results of the quenching of peptide in methanol by 2,3-dibromobutane are shown in Figure 1. Again, significant upward curvature is observed. The K_{SV} and V values determined for the quenching are 18.8 and 2.55 M^{-1}, respectively. Comparing these values with those for the quenching of indole by 2,3-dibromobutane, it can be seen that the Stern–Volmer rate constant is reduced by approximately a factor of 2, while the "static" quenching remains constant. These results demonstrate the collisional nature of the quenching process, with the peptide backbone effectively shielding the indole ring from quenching on one side. The "static" quenching, on the other hand, is unaffected by the presence of the peptide backbone and side chains. If the viscosity of the solvent is increased (i.e., by addition of glycerol), it can be seen that the collisional quenching constant K_{SV} for indole is decreased while the "static" quenching is again unchanged (Table I). These results differ from those of Eftink and Ghiron (1976), who found that the "static" component for the quenching of indole by acrylamide decreased with either an increase in viscosity or steric hindrance. Eftink and Ghiron also observed a value of r for the "sphere of action" that was 3–4 Å larger than the molecular radii of the quenching pair. They concluded that the quencher could diffuse those extra 3–4 Å during the lifetime of the fluorophore and, therefore, quenching appeared instantaneous. If this were the case, the "static" quenching should effectively be a collisional event, and, therefore, steric constraints and viscosity would be expected to affect the "static" component. Our results, on the other hand, do not show an affect of viscosity or steric hindrance on the "static" quenching. This suggests that "static" quenching, in this case, may occur without actual contact between the indole ring and the bromines.

Since the dynamic and "static" components for the quenching by 2,3-dibromobutane were obtained via a curve-fitting routine, it was desirable to confirm the value of the dynamic component (K_{SV}) by measurements of the fluorescent lifetime as a function of quencher concentration. For systems exhibiting both dynamic and "static" quenching, the dynamic portion of the quenching can be determined from the relation $\tau_0/\tau = 1 + K_{SV}[Q]$ (Lakowicz, 1983). Figure 2 shows the results of lifetime measurements on both indole and peptide in methanol as a function of 2,3-dibromobutane concentration. The slopes of the lines in Figure 2 are 35.3 and 22.2 M^{-1} for indole and peptide, respectively. These values compare well with the K_{SV}'s of 31.9 and 18.8 M^{-1} found for indole and peptide from F_0/F and confirm the dynamic nature of the quenching process.

Quenching of Peptide by Bromolipid. The quenching of tryptophanyl fluorescence by bromolipid was examined by incorporating the peptide into lipid vesicles made from the different bromolipids. Peptide was incorporated into lipid

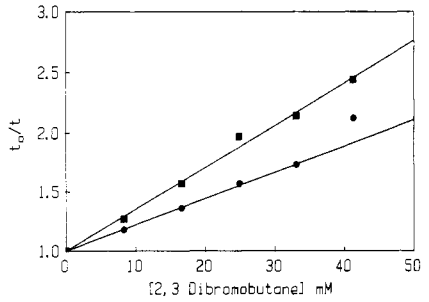

FIGURE 2: Measurements of the fluorescence lifetime of (■) indole or (●) peptide in methanol as a function of quencher (2,3-dibromobutane) concentration. τ is the fluorescence lifetime in the presence of quencher, and τ_0 is the fluorescence lifetime in the absence of quencher. Lifetime data for indole were described best by a monoexponential decay resulting in a single lifetime. Lifetime data for the peptide were described best initially by a diexponential, but converged toward a monoexponential as the concentration of quencher was increased. Therefore, all peptide data were force-fit to a monoexponential decay, and a single (average) lifetime was determined and used in the calculation of τ_0/τ.

Table II: Fluorescence Intensity of Peptide in Bromolipid Vesicles[a]

lipid	SUVs	LUVs
POPC	100	100
4,5-BRPC	78 ± 8	66 ± 3
6,7-BRPC	61 ± 5	53 ± 2
9,10-BRPC	39 ± 4	30 ± 2
11,12-BRPC	14 ± 2	11 ± 2
15,16-BRPC	10 ± 2	9 ± 1

[a] Fluorescence intensity measured at emission maximum. All fluorescence measurements were corrected for inner filter effects and peptide concentrations as described under Materials and Methods. The fluorescence is reported as a percentage of the fluorescence in POPC. Values for SUVs are averages of six independent measurements. Values for LUVs are the average of two independent measurements.

vesicles as described under Materials and Methods. The results of fluorescence measurements on the peptide–lipid vesicles are presented in Table II. The fluorescence is expressed as a percentage of the fluorescence observed for the peptide in POPC. As can be seen from Table II, the tryptophan is quenched significantly by bromines located either at the 15,16- or at the 11,12-position. This suggests that the tryptophan is located in the center of the bilayer as predicted. However, there is still significant quenching by bromines at the 9,10- and 6,7-positions, which are a considerable distance from a tryptophan supposedly located in the center of the bilayer. It is possible that not all of the peptide is bound in a transmembrane configuration as assumed, and so this possibility was tested by labeling the peptide–lipid vesicles with TNBS as described under Materials and Methods. If the peptide is orientated in a transmembrane configuration, the TNBS ratio (outer/total amino groups) should be 0.5. If the peptide is bound in a transmembrane configuration and additionally some peptide is bound to the surface of the vesicle, then the TNBS ratio will be greater than 0.5. TNBS labeling of peptide–lipid LUVs yielded a TNBS ratio of 0.46, indicating a transbilayer configuration. TNBS labeling of peptide–lipid SUVs, however, resulted in a TNBS ratio of 0.76, suggesting that some peptide was bound to the surface of the vesicles. The peptide–lipid SUVs were further characterized in order to determine the percentage of the peptide bound to the surface of the vesicles. Figure 3A,B shows the quenching of peptide–lipid SUVs by acrylamide. If peptide is bound to the surface, it should be accessible to quenching by acrylamide present in the aqueous phase. In this case, the results are expressed as $F_0/\Delta F$ versus $1/[Q]$ rather than F_0/F versus [Q]. This modification of the

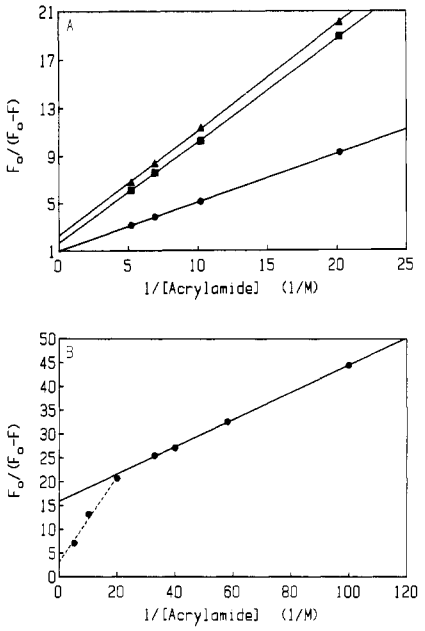

FIGURE 3: Fluorescence intensity of peptide–lipid SUVs as a function of acrylamide concentration. All samples were 0.5 mM in lipid. F and F_0 are the fluorescence intensities in the presence and absence of acrylamide, respectively. F and F_0 were corrected for scattering and inner filter effects as described under Materials and Methods. Symbols used for (A) are (●) peptide–15,16-BRPC, (■) peptide–9,10-BRPC, and (▲) peptide–6,7-BRPC. (B) shows the quenching for peptide–POPC vesicles.

Stern–Volmer relation was introduced to analyze the quenching of heterogeneous systems (Lehrer, 1971). This analysis should be applicable to the problem at hand, except in this case, we have a vesicle with a heterogeneous population, rather than a protein with a heterogeneous population. Peptide which is bound in a transmembrane configuration will have a tryptophan which is buried in the membrane and inaccessible to quenching by acrylamide. Peptide which is bound to the surface will have a tryptophan which is located on or near the surface of the membrane and therefore will be accessible to quenching by acrylamide. The inverse of the y intercept in Figure 3 yields the fraction of accessible fluorescence. For peptide–15,16-BRPC vesicles (Figure 3A), an intercept of 1 is found which indicates that all of the residual fluorescence (that fluorescence which has not been quenched by the bromine in the membrane) is accessible to acrylamide quenching. The intercepts found for peptide–9,10-BRPC vesicles and peptide–6,7-BRPC vesicles are 1.67 and 2.27, respectively. It can then be calculated that 60% of the residual fluorescence for the peptide–9,10-BRPC vesicles, and 44% of the residual fluorescence for the peptide–6,7-BRPC vesicles, is accessible to acrylamide. These results suggest that the peptide–lipid vesicles contain two populations of peptide. Since the residual fluorescence for the peptide–9,10-BRPC vesicles is 39% (value reported in Table I as percent of POPC fluorescence), it can be calculated that 23% (60% accessible × 39% residual) of the *total* fluorescence is accessible to acrylamide. Similarly, for the peptide–6,7-BRPC vesicles, 27% (44% accessible × 61% residual) of the total fluorescence is accessible to acrylamide. This suggests that approximately 25% of the peptide in the peptide–lipid SUVs is not in a transbilayer configuration. Figure 3B shows a plot of $F_0/\Delta F$ versus $1/[Q]$ for peptide–POPC SUVs. In this case, the plot appears to have two linear segments with different slopes. If the linear portion of the curve between 30 and 100 M^{-1} (low concentrations of acrylamide) is extrapolated to the y axis, an intercept of 15.9

is obtained. This corresponds to an accessible fraction of 6%. If the points between 0 and 20 M^{-1} are extrapolated, a y intercept of 4 is obtained, yielding an accessible fraction of 25%. These results again suggest that for the peptide–lipid SUVs, 25% of the peptide is bound on or near the surface of the vesicle. Since low concentrations of acrylamide can quench approximately 6% of the fluorescence, this suggest that some of the peptide must exist either in the aqueous phase or exposed on the vesicle surface. Chromatography of the peptide–lipid vesicles did not, however, affect the observed fluorescences, and, therefore, it is most likely that the peptide is bound to the surface rather than existing as a monomer in solution. It can be concluded then that the peptide exists in the following configurations in the peptide–lipid SUVs: 75% has a transmembrane configuration, 20% is bound to the surface such that the tryptophan is buried in the membrane but located close to the surface, and 5% of the peptide is bound to the surface such that the tryptophan is located on the surface exposed to the aqueous phase.

Theoretical Modeling of Fluorescence Quenching by Bromolipid. The acrylamide quenching results suggest that the peptide–lipid SUVs contain a mixed population of peptide configurations, yet the peptide distribution seen does not correspond to the quenching distribution observed. If the quenching by the bromolipids is accurately represented by 2,3-dibromobutane, then a close approach of the bromines to within a 10-Å radius of the tryptophan is all that is required for quenching to occur. A tryptophan located in the center of the bilayer should be quenched equally by the 15,16-BRPC and the 11,12-BRPC and not at all by the rest. If 20% of the peptide is bound to the surface with the tryptophan located in the membrane (but near the surface), then the residual fluorescence seen with the 9,10-BRPC, 6,7-BRPC, and 4,5-BRPC should be equivalent and should be ≈80%. It is possible that the peptide does not incorporate uniformly in each of the different bromolipids or that the high curvature of the SUVs causes significant displacement of the peptide and bromine from their theoretical positions in the bilayer. However, these problems should be reduced upon going from SUVs to LUVs, yet in the LUVs, the quenching by the 4,5-, 6,7-, and 9,10-BRPCs is higher not lower.

If the residual fluorescence (equivalent to F/F_0 with F_0 being the fluorescence of the peptide in POPC) is plotted as a function of the distance of the bromine position from the center of the bilayer, it appears that the quenching has a distance dependence (Figure 4). The effect of fluorophore–quencher separation on the efficiency of quenching has been well characterized for the case of dipole–dipole energy transfer in two dimensions. Surprisingly, the quenching of peptide by the different bromolipids can be fit quite reasonably assuming an r^6 dependence like that seen with energy transfer. The curve describing the fluorescence quenching F/F_0 as a function of distance (solid line) was constructed from the equations of Wolber and Hudson (1979) and Dewey and Hammes (1980) assuming a parallel infinite planes model, using approximant $A_{1,2}$. The best-fit curve was found to correspond to an R_0 of 9.25 Å. The experimental data were plotted assuming that the distance from the tryptophan to the bromines was equivalent to the distance from the center of the bilayer to the bromines as determined from X-ray diffraction (McIntosh & Holloway, 1987). As can be seen, for this value of R_0, the fluorescence of peptide in bromolipid LUVs is described quite well by the theoretical curve. The fluorescence of the peptide in SUVs does not fit as well, but does show approximately the same distance dependence. The deviations of the SUV data from the theoretical curve probably result from the presence of peptide on the surface of the vesicles, and the high curvature of the system. These results suggest that the quenching of tryptophan by dibrominated phospholipids involves some type of dipole–dipole interaction.

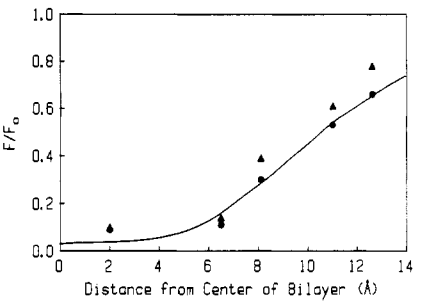

FIGURE 4: Fluorescence quenching as a function of distance between the tryptophan and bromine. It was assumed that the tryptophan was located in the center of the bilayer. Values for the positions of the bromines were taken from McIntosh and Holloway (1987). The solid line represents the quenching as a function of distance for dipole–dipole energy transfer in two dimensions and was calculated by the method of Wolber and Hudson (1979) and Dewey and Hammes (1980), approximant $A_{1,2}$, with an $R_0 = 9.25$ and $C = (1/71.7\ Å^2)(9.25\ Å)^2$. Values for F/F_0 for (●) peptide–lipid LUVs and (▲) peptide–lipid SUVs were taken from Table II.

CONCLUSIONS

In 1973, Berlman reported that quenching by brominated compounds required overlapping van der Waals radii. This observation was the basis for the later assumptions that quenching by bromolipid required contact between the fluorophore and the bromine. The results of the experiments presented here suggest that quenching by dibrominated compounds does not require contact as assumed. In addition, the results of the studies with indole and the different mono- and dibrominated hydrocarbons suggest that the efficiency of the quenching reaction is dependent on the bromination state of the compound. Surprisingly, the efficiency of the monobrominated butanes was only 7%, whereas the efficiency of the dibrominated compounds with the bromines located on adjacent carbon atoms was 80%. The study by Berlman was done with monobrominated compounds which we find have very low quenching rates, poor efficiencies, and negligible static quenching, and so this may explain his conclusion that overlapping van der Waals radii are required for quenching to occur.

The most surprising result from these studies was the observation that the quenching by the bromolipids had an r^6 distance dependence, suggesting that the quenching results from some type of dipole–dipole interaction. This result is difficult to understand in light of the results obtained for the quenching of indole by 2,3-dibromobutane in solution. These latter studies suggested that dynamic or collisional quenching was the dominant mechanism for the quenching reaction. A "static" component was found to contribute to the observed quenching, but oddly this "static" component was observed only with 2,3-dibromobutane and 1,2-dibromocyclohexane. If the "static" quenching were simply due to the fluorophore and quencher being in close proximity at the moment of excitation, then the "static" quenching for the different bromo compounds investigated here should be the same, since the probability of finding any of the different quenching molecules juxtapositioned next to an indole molecule at the moment of excitation would be the same. It could be argued that since the quenching efficiency of the monobromobutanes is so low, the "static" quenching is likewise low. However, 1,2-dibromo-

butane, which has the highest dynamic quenching rate, also has a negligible "static" quenching constant, suggesting that a specific molecular configuration is crucial for "static" quenching to occur.

In the membrane, the local concentration of bromine is very high (≈1.5 M; London & Feigenson, 1981). At this quencher concentration, assuming a value of 2.5 M^{-1} for the "static" quenching constant and a value of 18.8 M^{-1} for the collisional quenching constant (values of V and K_{SV} for the peptide in methanol), 98% of the observed quenching would be due to "static" interactions. Since the quenching in the membrane appears to result from some type of dipole–dipole interaction, it seems likely that the "static" quenching which was observed in solution is due to this same dipole–dipole interaction. This is consistent with the observation that the "static" quenching in solution was unaffected by an increase in viscosity or steric hindrance. This conclusion, on the other hand, appears to be at odds with the lifetime data, which showed that the so-called "static" component had no apparent affect on the lifetime. A dipole–dipole interaction would be expected to decrease the lifetime and increase K_{SV}. It is interesting to note, therefore, that the value of K_{SV} (35.3 M^{-1}) as determined by lifetime measurements was actually slightly *greater* than the value determined for K_{SV} (31.9 M^{-1}) by steady-state measurements. In solution, with the concentration of quencher used for the lifetime measurements (maximally 40 mM), approximately 10% of the observed quenching would be due to "static" interactions. Coincidently, the value of K_{SV} determined by lifetime measurements is 10% larger than that determined by steady-state measurements. This suggests that, within the limits of uncertainty associated with these measurements, the "static" component, in fact, has an effect on the lifetime.

If the details of the quenching by the bromolipids are examined, it is easy to show that quenching is significant only when the tryptophan and bromines are very close. Since R_0 was found to be ≈9 Å, the transfer efficiency would be 50% when the pair is separated by a distance of 9 Å. Since the distance of closest approach would be minimally 6–7 Å, "contact" is essentially required for efficient quenching to occur. The result of this is a quenching process which appears to be collisional in nature, but actually occurs through an energy-transfer mechanism. Regardless of the precise mechanism of quenching, it is apparent that the bromolipids are capable of accurately determining the position of a tryptophan residue in the membrane.

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