Distribution of Phospholipids around Gramicidin and D-β-Hydroxybutyrate Dehydrogenase As Measured by Resonance Energy Transfer†

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ABSTRACT: A resonance energy transfer method was developed to study the distribution of phospholipids around integral membrane proteins. The method involved measuring the extent of energy transfer from tryptophan residues of the proteins to different phospholipids labeled with a dansyl moiety in the fatty acid chain. No specific interactions were observed between gramicidin and dansyl-labeled phosphatidylcholine, phosphatidylethanolamine, or phosphatidic acid. The results were consistent with a random distribution of each phospholipid in the bilayer in the presence of gramicidin. However, a redistribution of both gramicidin and dansyl-labeled phospholipids was easily observed when a phase separation was induced by adding Ca^{2+} to vesicles made up of phosphatidylcholine and phosphatidic acid. Polarization measurements showed that in the presence of Ca^{2+} a rigid phosphatidic acid rich region and a more fluid phosphatidylcholine-rich region were formed. Energy-transfer measurements from gramicidin to either dansylphosphatidylcholine or dansylphosphatidic acid showed gramicidin preferentially partitioned into the phosphatidylcholine-rich regions. Energy-transfer measurements were also carried out with D-β-hydroxybutyrate dehydrogenase reconstituted in a vesicle composed of phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid. Although the enzyme has a specific requirement for phosphatidylcholine for activity, the extent of energy transfer decreased in the order dansylphosphatidic acid, dansylphosphatidylcholine, dansylphosphatidylethanolamine. Thus, the enzyme reorganized the phospholipids in the vesicle into a nonrandom distribution.

There is considerable evidence for the nonrandom distribution of lipids in biological membranes. Despite the dynamic nature of lipids, different membranes within a cell have different lipid compositions [e.g., endoplasmic reticulum, mitochondrial, and plasma membranes (Gilmore et al., 1979)]. A membrane may have an asymmetric distribution of phospholipids across the bilayer (Op den Kamp, 1979), and lipids within a membrane may be organized laterally into discrete regions or domains (Pessin & Glaser, 1980; Wolf et al., 1981; Bearer & Friend, 1982; Shukla & Hanahan, 1982; Karnovsky et al., 1982; Jain, 1983; Severs & Rubenek, 1983; Murphy & Woodrow, 1983; Thompson & Tillack, 1985). How these nonrandom distributions are generated and maintained are not understood. One possible mechanism is that integral membrane proteins could bind or sequester lipids and create a nonrandom distribution. Many physical studies on lipid–protein interactions have focused on the lipids immediately adjacent to the proteins, i.e., the boundary lipid or lipid annulus. In general, the boundary lipid appears not to be ordered and to exchange with the bulk lipid in the bilayer at an appreciable rate (approximately 10^6–10^7 s^{-1}) (Oldfield et al., 1978; Seelig & Seelig, 1978; Jost & Griffith, 1980; Smith & Oldfield, 1984). Depending on the on-rate and off-rate for binding of specific phospholipids to the protein, it seems feasible that the protein could enrich the area adjacent to it with specific phospholipids. The goal of the present investigaton was to test this hypothesis by using resonance energy transfer between tryptophan residues of either gramicidin or D-β-hydroxybutyrate dehydrogenase and specific labeled phospholipids.

Resonance energy transfer has proven to be a very useful method to study spatial relationships in a wide variety of macromolecular structures and biological assemblies (Stryer, 1978). A number of theoretical papers have treated the problem of energy transfer in two dimensions (Fung & Stryer, 1978; Wolber & Hudson, 1979; Estep & Thompson, 1979; Dewey & Hammes, 1980; Snyder & Friere, 1982; Doody et al., 1983; Kellerer & Blumen, 1984). Resonance energy transfer occurs over many angstroms and does not depend on direct contact between the protein and acceptor phospholipid, which is what is usually measured when protein–lipid interactions are studied by using techniques such as ESR or NMR. Also, the extent of resonance energy transfer should not be influenced by phospholipid exchange between the boundary lipid and bulk lipid since energy transfer is very rapid and occurs during the fluorescence lifetime (approximately 10 ns). Resonance energy transfer has been used, for example, to study the state of aggregation of the Ca^{2+}-ATPase (Vanderkooi et al., 1977) and the distance between the fluorescent tryptophan in cytochrome b_5 and *n*-(9-anthroyloxy) fatty acid probes in the membrane (Kleinfeld & Lukacovic, 1985). Fung and Stryer (1978) measured the extent of energy transfer between different fluorescent-labeled phospholipids to obtain information about the surface densities of the phospholipids in vesicles.

Initial experiments to determine the validity of the resonance energy transfer method to measure the distribution of phospholipids around membrane proteins were carried out with gramicidin, a polypeptide ionophore, whose interactions with phospholipids have been well characterized. Gramicidin inserts completely into the bilayer to form a dimer (Veatch & Stryer, 1977) and acts as a transmembrane channel. Gramicidin has no specific phospholipid requirement and preferentially partitions into liquid-crystalline regions as shown by quenching of gramicidin tryptophan fluorescence by spin-labeled phospholipids (Feigenson, 1983; London & Feigenson, 1981). D-β-Hydroxybutyrate dehydrogenase (BDH)[1] is one of the

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best-characterized enzymes that shows a specific phospholipid requirement (Fleischer et al., 1974). The enzyme can be purified free of phospholipids, but it is active only when it is reconstituted with phospholipids containing the choline head group (Isaacson et al., 1979; Grover et al., 1975).

The results of this study show that resonance energy transfer can be used to determine the distribution of phospholipids around membrane proteins. Gramicidin does not induce any phospholipid reorganization, while BDH can cause a nonrandom distribution of the phospholipids in a membrane.

Materials and Methods

Materials. Egg PC, egg PA, egg lyso-PC, bovine heart PC, *Escherichia coli* PE, phospholipase D (cabbage), gramicidin (*Bacillus brevis*, Dubos), DL-β-hydroxybutyrate, HEPES, DMPC, NAD^+, and TES were purchased from the Sigma Chemical Co. (St. Louis, MO). 11-Dansylundecanoic acid was purchased from Molecular Probes (Eugene, OR). Silica gel G plates (250 μm, 2.5 × 7.5 cm^2, and 500 μm, 20 × 20 cm^2) were from Analtech, Inc. (Newark, DE). Controlled-pore glass beads (CPG 10–350 120/200 mesh, mean pore diameters 369 Å) were purchased from Electro-Nucleonics (Bethesda, MD). Phospholipase A was obtained from *Naja naja siamensis* cobra venom (Miami Serpentarium, Miami, FL). Bovine serum albumin (fatty acid free, fraction V) was purchased from Miles Laboratories, Inc. (Elkhart, IN).

Synthesis of Dansyl-Labeled Phospholipids. Dansyl-PC was synthesized and purified by using the procedure for 11-(9-carbazolyl)undecanoic-L-α-phosphatidylcholine synthesis (Lackowicz & Hogen, 1980) except that the formation of the fatty acid anhydride was done at room temperature (Selinger & Lipidot, 1966) and all reactions employed toluene instead of benzene as the solvent (Omann & Glaser, 1984). The corresponding phosphatidylethanolamine (dansyl-PE) and phosphatidic acid (dansyl-PA) were formed from dansyl-PC by the action of phospholipase D as described by Yang et al. (1967). Dansyl-PE and dansyl-PA were purified by two-dimensional thin-layer chromatography on silica gel G plates. The first-dimension solvent was $CHCl_3/CH_3OH/NH_4OH$ (65:25:5), and the second-dimension solvent was $CHCl_3$/acetone/CH_3OH/acetic acid/H_2O (3:4:1:1:0.5). The labeled phospholipids had the same R_f values as unlabeled standards. The spots were scraped and eluted in $CHCl_3/CH_3OH/H_2O$ (10:20:1), and then the solvent was evaporated. The residue was extracted by the method of Bligh and Dyer (1959) to ensure complete removal of silica. The phospholipid to dansyl mole ratio was 1:1 as determined by dansyl absorbance and phospholipid phosphate analysis (Kates, 1972). The probes were stored at −20 °C in dry toluene.

Reconstitution of Gramicidin into Phospholipid Dispersions. Stock solutions of egg PC, egg PA, gramicidin, and dansyl-labeled phospholipids in organic solvents were mixed in the desired ratio and the solvents evaporated under a stream of nitrogen gas at 37 °C. Four milliliters of 10 mM HEPES, pH 7.0, and 100 mM KCl were added to each tube, and the tubes were capped under nitrogen. The dried lipid films on the bottoms of the tubes were hydrated at 37 °C for 15 min. The mixtures were dispersed by vigorous vortexing for 30 s and sonicating for 30 s with a bath sonicator. When 4.5 mM Ca^{2+} was added to induce a phase separation, the samples were quick frozen in liquid nitrogen, thawed at 37 °C, vortexed for 30 s, and sonicated for 30 s. One more such cycle was performed before measurements were taken in order to ensure full interaction of Ca^{2+} with the phospholipids.

Purification of BDH. The isolation of beef heart mitochondria followed the procedure of Blair (1967) with some slight modifications. The low-speed centrifugation step was carried out by using GS-3 and GSA rotors at 1600*g* for 10 min in a Sorvall RC-5B centrifuge (Clancy et al., 1981). The supernatant was further centrifuged in a GSA rotor at 12 500 rpm for 30 min. The mitochondrial pellet was resuspended in 0.25 M sucrose by using a 40-mL Dounce homogenizer. Release of BDH activity and purification of the enzyme followed the procedure described by Bock and Fleischer (1974). The purified protein was stored in 50 mM Tris-HCl, pH 8.0, 0.1 M KCl, 15 mM DTT, and 50% glycerol (v/v) at −75 °C. The standard BDH assay was carried out under the following conditions: 10 mM potassium phosphate buffer, pH 7.4, 0.5 mM EDTA, 0.04% BSA (w/v) (fatty acid free), 1.3% ethanol (v/v), 2 mM DTT, 2 mM NAD^+, appropriate amounts of dispersions of extracted beef heart mitochondrial lipids, and BDH. The mixture was preincubated at 25 °C for 15 min, and the reaction was started by adding 20 mM DL-β-hydroxybutyrate. The increase in absorbance at 340 nm was converted to units of activity by using an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NAD^+. When BDH activity was measured in mitochondria, an additional 0.5 μg/mL antimycin was present in the reaction mixture. Protein concentrations were determined as described by Lowry (1951) using BSA as a standard. In the presence of DTT the modified procedure described by Ross et al. (1973) was used instead.

Preparation of Vesicles for BDH Reconstitution. Mitochondrial lipids were extracted by the procedure described by Bligh and Dyer (1959). Dispersions of the lipids in 50 mM Tris-HCl, pH 7.4, were made by bath sonication for standard BDH activity assays. Veislces used for energy-transfer experiments were prepared by a sonication method similar to that described by Barrow et al. (1980). Equivalent amounts of beef heart PC, egg PA, and *E. coli* PE were mixed with desired amounts of dansylphospholipids, dried with nitrogen in small glass ampules, and put under vacuum overnight. A solution of 20 mM HEPES, 25 mM NaCl, and 1 mM EDTA, pH 7.2, that had been bubbled with N_2 was added into the ampules. The ampules were flushed with N_2 and sealed. The lipids were hydrated at 37 °C for 30 min, vortexed for 30 s, and sonicated for 7 min at 37 °C.

BDH, freshly dissolved DTT (0.5 mM), and the vesicles (200 nmol/mL) were mixed and incubated for 20 min at room temperature under N_2 in 20 mM HEPES, 25 mM NaCl, and 1 mM EDTA, pH 7.2. Before and after the fluorescence intensity was measured, small aliquots were taken to measure BDH activity by using the standard BDH assay. There was little loss of the activity during the fluorescence measurements. The absorbances of the samples at the excitation wavelength were less than 0.15.

Resonance Energy Transfer Measurements. Fluorescence intensity measurements were performed on an SLM 8000

[1] Abbreviations: BDH, D-β-hydroxybutyrate dehydrogenase; BSA, bovine serum albumin; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; dansyl-PC, 1-acyl-2-[11-[*N*-[5-(dimethylamino)naphthalene-1-sulfonyl]amino]undecanoyl]phosphatidylcholine; dansyl-PA, 1-acyl-2-[11-[*N*-[5-(dimethylamino)naphthalene-1-sulfonyl]amino]undecanoyl]-phosphatidic acid; dansyl-PE, 1-acyl-2-[11-[*N*-[5-(dimethylamino)-naphthalene-1-sulfonyl]amino]undecanoyl]phosphatidylethanolamine; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; egg lyso-PC, lysophosphatidylcholine from egg yolk; PC, phosphatidylcholine; PA, phosphatidic acid; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PE, phosphatidylethanolamine.

fluorometer in the ratiometric and integrating mode. The proteins were excited at 282 nm. The band width of the excitation light was 2 nm. A Corning 7-54 filter was used in the incident light path. The tryptophan fluorescence was observed through Schott WG345 and Corning 7-60 filters, and the dansyl fluorescence was observed through a Corning 3-73 filter and a 1-mm filter filled with 2 M $NaNO_2$. Measurements were carried out at 25 °C. Unlabeled vesicles were always used to subtract the scattering background. The energy-transfer efficiency can be calculated from either the tryptophan fluorescence quenching data or the dansyl fluorescence enhancement data. The energy-transfer efficiency was calculated from the tryptophan data by using

$$E = 1 - F/F_0 \quad (1)$$

where F_0 is the fluorescence intensity of tryptophan in the absence of the energy-transfer acceptor and F is the fluorescence intensity of tryptophan in the presence of the energy-transfer acceptor, a dansyl-labeled phospholipid. The energy-transfer efficiency was calculated from the dansyl enhancement data by using

$$E = (\text{slope} \times \epsilon_a \times \sigma_a)/\epsilon_d \quad (2)$$

where ϵ_a and ϵ_d are the extinction coefficients of the energy-transfer acceptor and donor at the excitation wavelength, respectively, and σ_a is the density of the acceptor (acceptor to phospholipid mole ratio) (Fung & Stryer, 1978). The extinction coefficients of gramicidin and the dansyl-labeled phospholipids were determined to be 20 000 and 2100 M^{-1} cm^{-1}, respectively, at 282 nm. The extinction coefficient of BDH at 282 nm was assumed to be the same as that of gramicidin, since there are four tryptophans in both BDH and gramicidin. The slope was determined from a plot of F/F_0 vs σ_d, where F and F_0 are the dansyl emission intensities in the presence and absence of the energy-transfer donor, respectively, and σ_d is the surface density of the donor in the membrane.

Fluorescence Spectra, Polarization, Lifetime, and Quantum Yield. Excitation and emission spectra of dansyl-labeled phospholipids, BDH, and gramicidin were taken on a photon-counting fluorometer. All the spectra were corrected for wavelength-dependent effects of the instrument. Steady-state polarization and quantum yield measurements were taken on a SLM 8000 fluorometer. The fluorescence lifetimes of dansyl-labeled phospholipids were measured by using a phase and modulation fluorometer as described by Gratton and Limkeman (1983). Tryptophan with a quantum yield of 0.2 in 10 mM HEPES, pH 7.0, was used as the standard to measure the quantum yield of gramicidin (Teale & Weber, 1957); 2.5% (mol/mol) gramicidin in egg PC/egg PA vesicles at a total phospholipid concentration of 250 nmol/mL was used for the measurements. The quantum yield was calculated by using

$$Q_1/Q_2 = [F_1/(1 - 10^{-A_1})]/[F_2/(1 - 10^{-A_2})] \quad (3)$$

where Q_1 and Q_2 are the quantum yields, F_1 and F_2 are the tryptophan fluorescence intensities, and A_1 and A_2 are the absorbances of the standard tryptophan solution and gramicidin, respectively. Tryptophan and gramicidin were excited at 282 nm.

RESULTS

Gramicidin was chosen for the initial experiments in order to determine the feasibility of using resonance energy transfer to measure the distribution of phospholipids around membrane proteins. The tryptophan fluorescence of gramicidin was proportional to the amount of the protein in the membrane up to a protein to phospholipid ratio of approximately 0.2 (data not shown). At higher gramicidin concentrations the fluorescence decreased. Since substantial fluorescence intensity was observed at much lower concentrations, measurements were routinely made below a gramicidin to phospholipid ratio of 0.02.

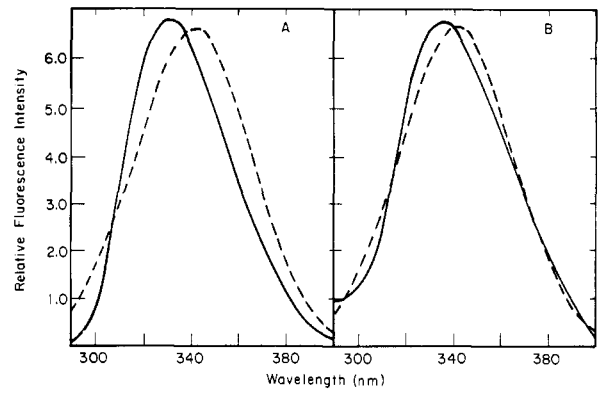

FIGURE 1: Fluorescence emission spectra of gramicidin (A) and BDH (B) compared to the excitation spectra of dansyl-PC. Dansyl-PC was mixed with egg PC/egg PA (2:1 mol/mol) at a ratio of 100:1 and a total lipid concentration of 200 nmol/mL. Gramicidin was reconstituted with egg PC/egg PA (2:1 mol/mol) at a lipid to protein mole ratio of 150:1 and a lipid concentration of 200 nmol/mL. BDH was reconstituted in beef heart PC/*E. coli* egg PA (1:1 mol/mol) vesicle with a lipid to protein mole ratio of 24:1 and a total lipid concentration of 400 nmol/mL. (---) Normalized excitation spectra of dansyl-PC (A and B); (—) emission spectra of gramicidin (A) and BDH (B). The excitation wavelength for the proteins was 282 nm, and the emission wavelength for dansyl-PC was 500 nm.

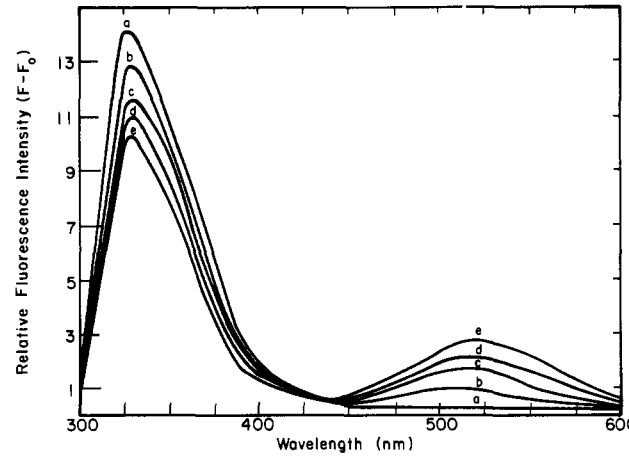

FIGURE 2: Fluorescence emission spectra of gramicidin and dansyl-PC in egg PC/egg PA vesicles. The phospholipid concentration was 200 nmol/mL (egg PC/egg PA 2:1 mol/mol). The lipid to gramicidin mole ratio was constant (150:1). The lipid to dansyl-PC mole ratio was 330:0 in (a), 330:1 in (b), 330:2 in (c), 330:3 in (d), and 330:4 in (e). The excitation wavelength was 282 nm. The emission of samples containing equivalent amounts of dansyl-PC but without gramicidin was subtracted from the spectra.

The fluorescence emission spectra of gramicidin and BDH are compared to the excitation spectra of dansyl-PC in Figure 1. There was substantial overlap between the tryptophan fluorescence spectra of these proteins and the excitation spectra of dansyl-PC, making them good donor and acceptor pairs for energy-transfer experiments. The excitation spectra of dansyl-PC and dansyl-PA in the presence or absence of 4.5 mM Ca^{2+} were all similar (not shown).

The result of energy transfer on the fluorescence spectra of gramicidin and dansyl-PC is illustrated in Figure 2. As the concentration of dansyl-PC was increased, there was a decrease in the tryptophan fluorescence intensity and a parallel

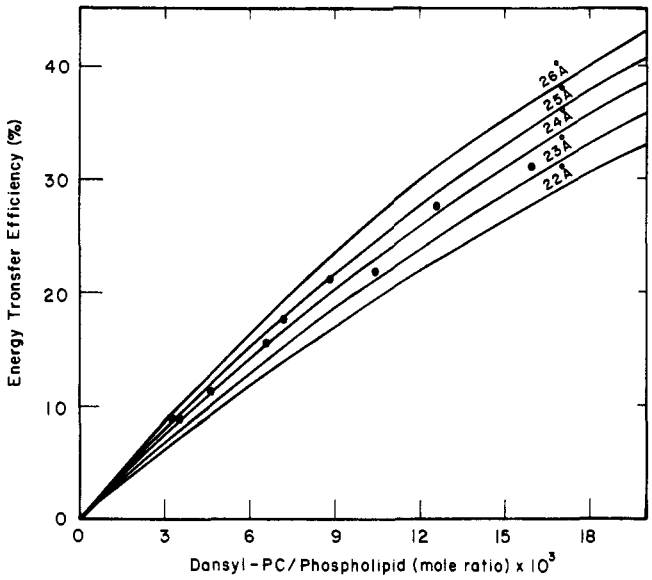

FIGURE 3: Efficiency of energy transfer from gramicidin to dansyl-PC as a function of dansyl-PC/phospholipid ratio. The phospholipid concentration was 190 nmol/mL (egg PC/egg PA 2:1 mol/mol). The phospholipid to gramicidin mole ratio was 140:1. The samples were excited at 282 nm, and the tryptophan fluorescence was measured. The experimental points (●) were calculated from the tryptophan quenching data, and the solid curves were calculated for a random array of donors and acceptors with R_0 = 22, 23, 24, 25, and 26 Å (see text for details). A R_0 = 24 ± 1 Å gave the best fit to the experimental data.

enhancement of the dansyl fluorescence intensity, clearly showing the dependence of energy transfer on the surface density of the acceptor.

The distance between the donor and acceptor that results in a 50% energy-transfer efficiency, R_0, can be calculated by using

$$R_0^6 = (8.785 \times 10^{-25})(J\kappa^2 n^{-4} Q_D) \quad (4)$$

where J is the overlap integral between the emission spectrum of the donor and the absorption spectrum of acceptor, κ^2 is the orientation factor for the donor and acceptor, n is the refractive index of the medium, and Q_D is the fluorescence quantum yield of the donor in the absence of the acceptor. κ^2 was assumed to be $^2/_3$, and n was assumed to be 1.4. Q_D for gramicidin in phospholipid vesicles was determined to be 0.11. J was determined from the fluorescence emission of the protein and the excitation of the dansyl-PC (Figure 2) by using

$$J = \int \epsilon_a(\omega) f(\omega) \omega^4 \, d\omega \quad (5)$$

where $\epsilon_a(\omega)$ is the extinction coefficient of dansylphospholipids at wavelength ω and $f(\omega)$ is the fractional fluorescence intensity of the protein at wavelength ω. The absorption spectrum was normalized by using a measured extinction coefficient of 4300 M^{-1} cm^{-1} at 335 nm. Since gramicidin and BDH had similar emission spectra, the value of R_0 was calculated to be 22 Å for both gramicidin and BDH.

The extent of energy transfer can be measured by determining either the extent of tryptophan quenching or the extent of fluorescence enhancement of the dansyl moiety. The dependence of energy-transfer efficiency on surface density of the acceptor dansyl-PC is shown in Figure 3. The data represent the extent of tryptophan quenching and are best fit to a theoretical curve calculated for a random planar array of donors and acceptors with R_0 = 24 ± 1 Å according to the theory of Fung and Stryer (1978). Since the fit is good, it suggests that dansyl-PC is randomly distributed around the gramicidin molecules.

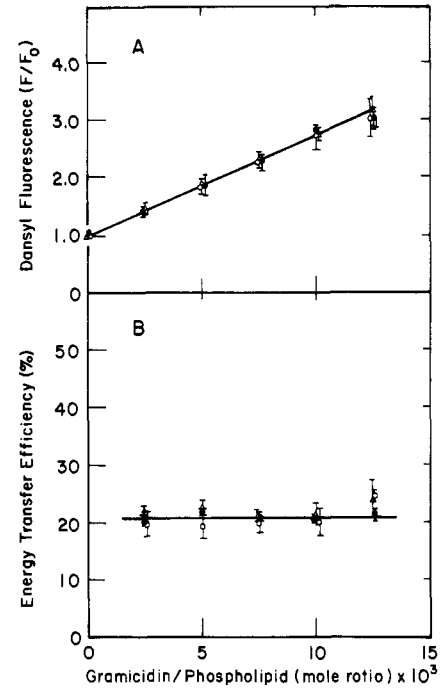

FIGURE 4: Energy transfer from gramicidin to dansyl-PC (●), dansyl-PA (△), and dansyl-PE (○). The samples were excited at 282 nm, and both the dansyl fluorescence enhancement (A) and the tryptophan fluorescence quenching (B) were measured. The energy-transfer efficiencies were calculated from the tryptophan fluorescence quenching data by using eq 1. The phospholipid concentration was 150 nmol/mL with a 1:1:1 mole ratio of egg PC/egg PA/egg PE. The dansyllipid to phospholipid mole ratio was 0.01.

The extent of energy transfer can also be measured by the increase in dansyl fluorescence. The increase in dansyl fluorescence corresponded to the amount of gramicidin in the bilayer (Figure 4A). The extent of energy transfer between gramicidin and different acceptors, either dansyl-PC, dansyl-PE, or dansyl-PA, was similar. In this experiment the overall phospholipid composition of the vesicles was the same and only the type of dansyl-labeled phospholipid was varied (approximately 1 mol %). This experiment indicates that the distances between gramicidin and the different dansyl-labeled phospholipids were the same and there was no selective enrichment of a certain phospholipid around the gramicidin molecules.

The extent of dansyl fluorescence enhancement was used to calculate the energy-transfer efficiency by using eq 2. The energy-transfer efficiency calculated this way was approximately 20%. It agreed well with the calculations of the energy-transfer efficiency, approximately 21%, from eq 1 and measurements of tryptophan quenching (Figure 4B). The results show that the energy-transfer efficiency was independent of the surface density of the donor as predicted by theory (Fung & Stryer, 1978).

When a phase separation was induced by the addition of a divalent cation to a vesicle containing phosphatidic acid, it was observed that gramicidin would partition preferentially into the fluid phase (Feigenson, 1983). Consequently, it was of interest to determine if the energy-transfer methods used in this study would be a good way to measure the unequal partitioning of gramicidin in the different phases. Steady-state polarization of the dansyl moiety readily illustrated the phase separation caused by Ca^{2+} in egg PA/egg PC mixtures as shown in Figure 5. Without Ca^{2+}, egg PC and egg PA formed a fluid phase as indicated by the low polarization values (approximately 0.1) for dansyl-PC and dansyl-PA. When Ca^{2+} was added, there was almost no change in the dansyl-PC

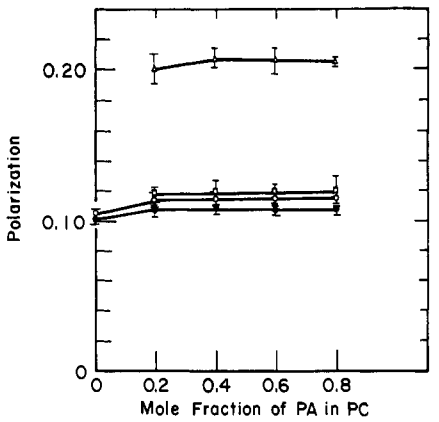

FIGURE 5: Steady-state polarization of dansyl-PC and dansyl-PA in egg PC/egg PA vesicles in the presence and absence of Ca^{2+}. The phospholipid concentration was 190 nmol/mL, and the phospholipid to dansyl-PC or dansyl-PA mole ratio was 220:1. Samples were egg PC/egg PA with dansyl-PC (●), dansyl-PA (□), dansyl-PC plus 4.5 mM Ca^{2+} (○), and dansyl-PA plus 4.5 mM Ca^{2+} (△). The excitation wavelength was 350 nm.

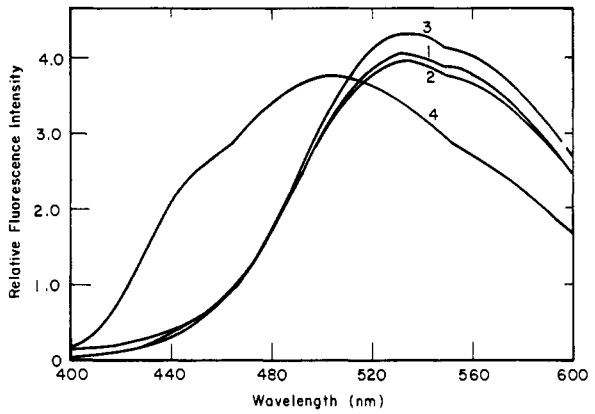

FIGURE 6: Emission spectra of dansyl-PC (1), dansyl-PA (2), dansyl-PC plus 4.5 mM Ca^{2+} (3), and dansyl-PA plus 4.5 mM Ca^{2+} (4) in vesicles containing 200 nmol/mL of egg PC and egg PA (mole ratio 2:1) plus 1 mol % dansyl-PC or dansyl-PA. The samples were excited at 350 nm.

polarization value, but there was a significant increase in the dansyl-PA polarization. The emission spectra of dansyl-PC and dansyl-PA were similar without Ca^{2+} (Figure 6). Addition of Ca^{2+} increased the emission intensity of dansyl-PC slightly, but the maximum emission wavelength was not changed. However, there was a blue shift in the dansyl-PA emission spectrum when Ca^{2+} was present, indicating a more nonpolar environment around the dansyl-PA molecules. To exclude the effect of a change in the fluorescence lifetime on the observed polarization change, lifetimes of dansyl-PC and dansyl-PA were measured in egg PC/egg PA vesicles at a mole ratio of 2:1 in the absence and presence of Ca^{2+}. In the absence of Ca^{2+} dansyl-PC had an average lifetime of 12.0 ns and dansyl-PA 11.9 ns, and in the presence of 4.5 mM Ca^{2+} the average lifetime of dansyl-PC was 14.0 ns and that of dansyl-PA 15.0 ns. Since an increase only in the lifetime would cause a decrease in polarization, the results are consistent with Ca^{2+} causing a phase separation by clustering the negatively charged phospholipids into a more rigid PA-rich region as has been established previously (Ito et al., 1975; Van Dijck et al., 1978; Hoekstra, 1982; Feigenson, 1983; Tanaka & Schroit, 1986; Haverstick & Glaser, 1987).

When gramicidin was introduced into the egg PC/egg PA vesicles in the absence of Ca^{2+}, the extent of energy transfer between gramicidin and either dansyl-PC or dansyl-PA was

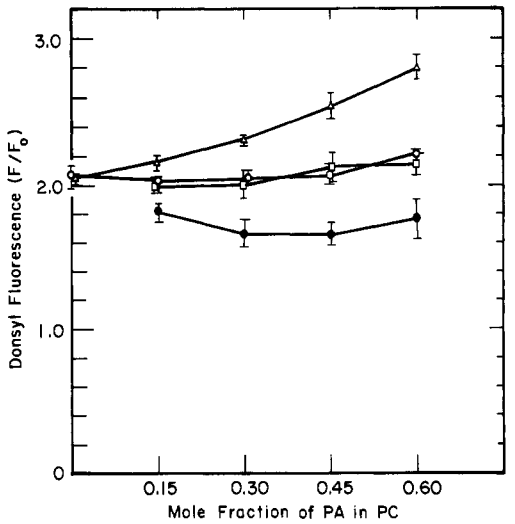

FIGURE 7: Energy transfer from gramicidin to dansyl-PC and dansyl-PA in the presence and absence of Ca^{2+}. The fluorescence intensity of dansyl was monitored with excitation at 282 nm. The phospholipid concentration was 65 nmol/mL, the dansylphospholipid to phospholipid mole ratio was 2:100, and the gramicidin to lipid mole ratio was 0.7:100. Samples were dansyl-PC with 4.5 mM Ca^{2+} (△), dansyl-PA (□), dansyl-PC (○), and dansyl-PA with 4.5 mM Ca^{2+} (●).

similar (Figure 7). This was consistent with gramicidin and both acceptors being randomly distributed in the membranes. When Ca^{2+} was added, the extent of energy transfer was much greater to dansyl-PC than to dansyl-PA, showing that the distribution of the two phospholipids around gramicidin was different. Gramicidin preferentially partitioned into the PC-rich region and was excluded from the more rigid PA-rich region.

To check orientation (κ^2) effects on energy transfer, the transfer efficiencies between gramicidin and dansyl-PC were measured at several temperatures. No significant changes (<3%) were observed when the temperature was varied from 8 to 43 °C. This indicated that the rotational motions of the donor and acceptor were sufficiently rapid at low temperature so that an increase in temperature did not further randomize the orientation between the donor and the acceptor. In addition, the steady-state polarization of gramicidin and dansyl-labeled PC and PA were measured to estimate the extent of their motion in the membranes. In a vesicle containing unlabeled egg PC and egg PA (55:45 mole ratio) and 1 mol % gramicidin, the tryptophan polarizations were 0.127 and 0.145 when excited at 300 nm in the absence and presence of 4.5 mM Ca^{2+}, respectively. These values indicate a large rotational freedom of the tryptophan in the membrane bilayer since they are much smaller than the limiting polarization of tryptophan [approximately 0.40 at this excitation wavelength (Valeur & Weber, 1977)]. The steady-state polarizations of the dansyl-labeled phospholipids shown in Figure 5 also indicate rapid rotational motion of the dansyl group (the limiting polarization of the dansyl group at an excitation wavelength of 350 nm is 0.44). An analysis by Haas et al. (1978), for example, concluded that small polarization values for the donor and acceptor molecules result in only a small error in the estimation of distances from energy-transfer measurements due to orientation effects. The error is not likely to exceed 10% for polarizations smaller than 0.3. Since the polarization values in this study were significantly smaller than 0.3, the data indicate that a random orientation ($\kappa^2 = {}^2/_3$) satisfactorily describes the donor–acceptor orientational distribution.

BDH was also used as the energy-transfer donor to the different dansyl-labeled phospholipids. In contrast to the

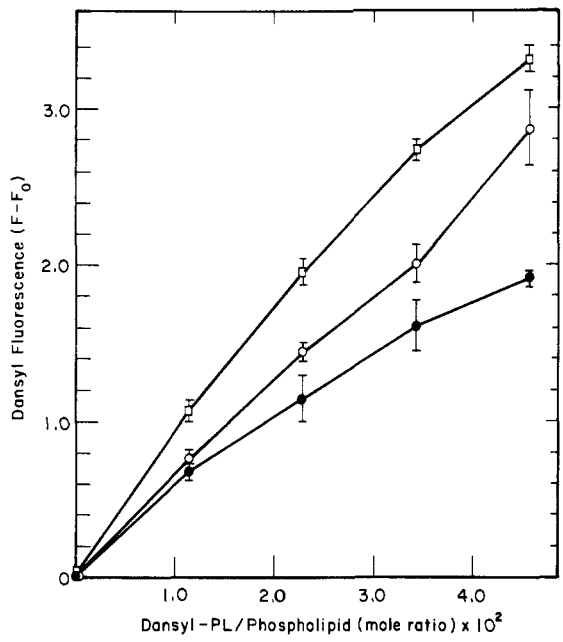

FIGURE 8: Energy transfer from BDH to dansyl-PC (○), dansyl-PA (□), and dansyl-PE (●). BDH was reconstituted with vesicles composed of beef heart PC/*E. coli* PE/egg PA (1:1:1 mole ratio) and variable amounts of dansyl-labeled phospholipids. The lipid to protein mole ratio was 120:1, and the total phospholipid concentration was 200 nmol/mL. The detailed method used for reconstitution is described under Materials and Methods. The excitation wavelength was 282 nm, and the fluorescence of dansyl was measured.

results with gramicidin, the extent of energy transfer was different for each phospholipid as shown in Figure 8. The greatest extent of energy transfer was observed with dansyl-PA and the least with dansyl-PE. The extent of energy transfer with dansyl-PC was intermediate. No energy transfer could be detected when BDH was added to the vesicles under conditions where no reconstitution took place, e.g., high pH. That is, effective reconstitution that indicates proper binding to the vesicles was necessary for energy transfer.

Discussion

Resonance energy transfer provides a sensitive method for studying the distributions of phospholipids around membrane proteins. As illustrated by the experiments with gramicidin, measurements can easily be made with as low as 0.3 mol % gramicidin and 0.3 mol % acceptor in a total lipid concentration of 60 μM. Other physical methods used to examine lipid–protein interactions usually require much higher concentrations.

Similar extents of energy transfer were observed between gramicidin and the different phospholipid acceptors. The results agreed with a theoretical calculation assuming a random distribution of acceptors around the donor (Figure 3). When a phase separation was induced by Ca^{2+} in a PA/PC mixture, the redistribution of phospholipids around gramicidin was readily apparent (Figure 7). In contrast to gramicidin, BDH showed a selectivity for particular phospholipids (Figure 8). The different extents of energy transfer between BDH and the different phospholipids were similar to the differences in energy transfer between gramicidin and dansyl-PA and dansyl-PC when a phase separation was induced with Ca^{2+}. In the case of gramicidin, however, the distribution of phospholipids within a phase probably was random, and the differences in energy transfer reflected the different partioning of gramicidin into the PC-rich rather than the PA-rich phase. In the case of BDH, the phospholipid distribution was nonrandom with the protein enriching its environment with a particular phospholipid (i.e., PA > PC > PE). Interestingly, the efficiency of BDH reconstitution into phospholipid vesicles is much higher if a negatively charged phospholipid is present, although the negatively charged phospholipid alone will not restore enzyme activity (Churchill et al., 1983).

Further research is necessary to quantitatively fit the data to a particular model for the phospholipid distribution. BDH is a tetramer with four identical subunits and four tryptophans per subunit (McIntyre et al., 1983). BDH penetrates into the hydrocarbon region to some extent (Maurer et al., 1985), but the three-dimensional structure and the exact degree of penetration of the protein into the bilayer is not known. If the situation is oversimplified by assuming a single tryptophan (with 4 times the extinction coefficient) and a planar array of donors and acceptors with a closest approach of 8 Å, then the data for BDH energy transfer to a given phospholipid (Figure 8) can be fit by a random distribution of acceptors. When BDH was added to an equimolar mixture of PA, PC, and PE, the reorganization of the phospholipids would be equivalent to a concentration of 49% PA, 32% PC, and 20% PE.

BDH has a specific requirement for PC and only a few molecules per monomer are necessary to activate the enzyme (Cortese et al., 1982). These PC molecules exchange with the bulk lipid in the bilayer faster than the turnover time of the enzyme (milliseconds) (Clancy et al., 1983). To explain the energy-transfer results with BDH, it can be postulated that the enzyme has multiple phospholipid binding sites in addition to the PC site(s) necessary for activation. These other sites would have a higher affinity for PA than for PC. The phospholipids in these sites would exchange with the bulk phospholipids in the membrane, but the exchange would be slow, so that the area adjacent to the enzyme would be enriched with PA. PE would have the least affinity for the enzyme, and its concentration adjacent to the enzyme would be the lowest.

This model is consistent with studies on the phospholipid dependence of BDH in vivo. When the phospholipid composition of primary rat hepatocyte cultures was manipulated, there was a lag before the change in phospholipid composition was reflected in the activity of BDH (Clancy et al., 1983). It was postulated that BDH existed in a localized lipid region that did not change its composition as rapidly as the bulk lipid in the membrane. A mechanism for this could be the existence of phospholipid binding sites on BDH that cause an enrichment of certain phospholipids in the vicinity of the protein. This type of mechanism could give rise to domains or localized lipid regions on a larger scale and explain how enveloped viruses, for example, acquire a distinct lipid composition (Pessin & Glaser, 1980).

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