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Thermotropic and Dynamic Characterization of Interactions of Acylated α-Bungarotoxin with Phospholipid Bilayer Membranes†

Bruce Babbitt, Leaf Huang, and Ernesto Freire*

ABSTRACT: The interactions of palmitoyl-α-bungarotoxin (PBGT) with dipalmitoylphosphatidylcholine (DPPC) bilayers have been studied by using high-sensitivity differential scanning calorimetry together with steady-state and time-resolved phosphorescence and fluorescence spectroscopy. The incorporation of PBGT into large single lamellar vesicles causes a decrease in the phospholipid phase transition temperature (T_m), a broadening of the heat capacity function, and a decrease in the enthalpy change associated with the phospholipid gel to liquid-crystalline transition. Analysis of the dependence of this decreased enthalpy change on the protein/lipid molar ratio indicates that each PBGT molecule exhibits a localized effect upon the bilayer, preventing approximately six lipid molecules from participating in the lipid phase transition. Additional calorimetric experiments indicate that binding to acetylcholine receptor enriched membranes causes a small increase in the T_m of the PBGT/DPPC vesicles. Steady-state fluorescence depolarization measurements employing 1,6-diphenyl-1,3,5-hexatriene (DPH) indicate that the association of PBGT with the phospholipid bilayer decreases the apparent order of the bulk lipid below T_m while increasing the order above T_m. These results have been further supported by rotational mobility measurements of erythrosin-labeled PBGT associated with giant (about 2-μm) unilamellar vesicles composed of dielaidoylphosphatidylcholine or dioleoylphosphatidylcholine using the time-dependent decay of delayed fluorescence/phosphorescence emission anisotropy. Rotational correlation times in the submillisecond time scale (about 30 μs) indicate that the protein is highly mobile in the fluid phase and that below T_m the rotational mobility is only slightly restricted. Lateral diffusion measurements of fluorescein-labeled PBGT ($PBGT^F$) associated with multilamellar liposomes composed of egg PC or DPPC measured by fluorescence recovery after photobleaching technique indicate that the lateral mobility of $PBGT^F$ is dependent upon the bilayer fluidity. Lateral diffusion coefficients at 25 °C of $(1.7 \pm 1.2) \times 10^{-8}$ cm^2/s (egg PC) and 1×10^{-11} cm^2/s (DPPC) were obtained. These results are consistent with a model in which PBGT interacts with the phospholipid bilayer principally via the fatty acid chain while the polypeptide region of the molecule does not interact strongly with the bilayer.

Specific interactions between membranes are of fundamental importance in many biological processes, including cellular recognition, immune responses, cellular adhesion and sorting, cell fusion, fertilization, and neuronal recognition. Presently, however, very little is known regarding the molecular details of these processes as well as the effects of membrane–membrane interactions mediated by specific ligand–receptor pairs on the physical and functional properties of the two adjacent membranes.

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We have previously developed a model system to study membrane interactions mediated by specific ligand–receptor binding (Grant et al., 1982). For that purpose we chose the binding of nicotinic acetylcholine receptor enriched plasma membrane vesicles (microsacs) to liposomes containing incorporated acylated α-bungarotoxin. Even though α-bungarotoxin is a water-soluble polypeptide of M_r 8000, the attachment of a single palmitic acid alters dramatically its water solubility and allows total incorporation into phospholipid bilayer vesicles without appreciable loss of its biological activity (Grant et al., 1982). Since the interactions between αBGT[1] and the acetylcholine receptor have been well characterized from a biochemical and biophysical point of view, this ligand–receptor pair constitutes an ideal model system with which to study ligand–receptor-mediated membrane–membrane interactions.

In recent years it has become apparent that many membrane proteins from sources as diverse as enveloped viruses, tissue culture cells, and animal cells contain covalently bound lipid usually in the form of a long-chain, saturated fatty acid ester linked to various hydroxy amino acids of the protein (Magree & Schlesinger, 1982). For example, VSVG glycoprotein, Sindbis virus E1 and E2 glycoproteins, brain myelin proteolipoprotein, human transferrin receptor, the proteolipid subunit of the Mg^{2+}- and Ca^{2+}-ATPase of sarcoplasmic reticulum, human histocompatibility antigen (HLA), and the $p60^{SRC}$-transforming protein of Rous sarcoma virus, to name a few, have all been shown to be acylated membrane proteins containing covalently bound fatty acid in a domain of the molecule that interacts strongly with the lipid bilayer. However, up to this point in time the function of the covalently bound lipid is not understood although speculation exists that it may play a role in intracellular transport of membrane proteins, initiation or stabilization of protein insertion into membranes, and various modes of intercellular or intracellular membrane fusion (including virus budding). Therefore, the study of the physical interaction of monoplamitoyl-α-bungarotoxin (PBGT) with model membrane bilayers should provide useful information concerning the role of the fatty acid in the structure/function of an acylated membrane protein.

In this paper high-sensitivity differential scanning calorimetry, steady-state and time-resolved fluorescence spectroscopy, and fluorescence recovery after photobleaching (FRAP) have been used to determine the effect of incorporated α-bungarotoxin on the thermotropic behavior of phospholipid vesicles, the rotational and lateral mobility of the acylated protein on the membrane surface, and the interactions of these vesicles with plasma membranes highly enriched with the acetylcholine receptor.

Experimental Procedures

Materials

Dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), dielaidoylphosphatidylcholine (DEPC), and egg lecithin were purchased from Avanti Biochemicals (Birmingham, AL) and used without further purification. α-Bungarotoxin (αBGT) was purified from the lyophilized venom of *Bungarus multicinctus* (Miami Serpentarium, Miami, FL) as previously reported (Lee et al., 1972) and iodinated with Chloramine T (Hunter & Greenwood, 1962). The toxin samples used in these experiments were found to contain a single band of 8000 daltons when electrophoresed on a 12% SDS–polyacrylamide gel. All experiments were performed in the presence of 2.5 mM EGTA to inhibit any phospholipase activity in these preparations. Erythrosin (tetraiodofluorescein) isothiocyanate was purchased from Molecular Probes, Inc. (Junction City, OR) and used without further purification. Stock solutions of erythrosin isothiocyanate in anhydrous acetone were stored in the dark at −20 °C. The *N*-hydroxysuccinimide ester of palmitic acid (NHSP) was synthesized according to Lapidot et al. (1967) and recrystallized. Palmitic acid was covalently coupled to αBGT as previously reported (Grant et al., 1982). This procedure results in a 70% yield of monopalmitoyl-αBGT (PBGT), which was dissolved and stored in 50% EtOH at −20 °C. Acetylcholine receptor rich membrane vesicles (microsacs) were prepared from the electroplax tissue of *Torpedo californica* (Pacific Biomarine, Venice, CA) as previously described (Miller et al., 1978).

Methods

Covalent Labeling of PBGT. Seventy-four nanomoles of radiolabeled ^{125}I-PBGT in a final volume of 500 μL in 3.2% deoxycholate and 86 mM $NaHCO_3$ (pH 9) was added to a dry film of erythrosin isothiocyanate (1.85 μmol) and incubated for 2 h at room temperature. The reaction mixture was applied to a 20-mL Sepharose LH-20 column preequilibrated in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1 mM Na_2HPO_4) at pH 8.0. The void volume containing erythrosin-labeled monopalmitoyl-αBGT ($PBGT^E$) was then applied to a 1-mL phenyl-Sepharose column preequilibrated with PBS at pH 8.0. The column was first washed with PBS and then eluted with 50% EtOH in distilled water and extensively dialyzed in a Spectrapor-6 dialysis membrane for 48 h against 500 mL of 50% EtOH in distilled water. The stoichiometry of the final reaction product was found to be one molecule of erythrosin probe covalently bound per molecule of PBGT when an extinction coefficient of 83 mM^{-1} cm^{-1} at 540 nm for the attached erythrosin moiety in PBS was employed. In order to label PBGT with fluorescein isothiocyanate (FITC), a 1 mg/mL solution of PBGT in PBS containing 0.2% deoxycholate (DOC) was mixed with an equal volume of 1 M $NaHCO_3$ buffer (pH 8.5) containing 0.2 mg of FITC/mL and 0.2% DOC. The mixture was incubated at 4 °C in the dark for 4.5 h and then chromatographed on a Bio-Gel P-4 column. FITC-labeled PBGT ($PBGT^F$) was eluted in the void volume fractions with PBS containing 0.2% DOC. This labeling procedure did not cause any significant detachment of the palmitic acid, since $PBGT^F$ was precipitated upon dialysis in detergent-free PBS.

Vesicle Preparations. (1) FUV. Fused unilamellar vesicles were prepared as described previously (Freire et al., 1983). DPPC was dried from a chloroform solution, lyophilized overnight, and suspended by vortexing in 50 mM KCl containing 0.02% sodium azide to a final concentration of 70 mM. Lipid suspensions were sonicated and centrifuged above the lipid phase transition temperature and then incubated at 4 °C for at least 1 week as described by Wong et al. (1982). This low-temperature incubation triggers a spontaneous fusion process and produces a homogeneous population of single lamellar vesicles of about 900-Å diameter (Wong et al., 1982).

(2) Giant Vesicle Formation. Giant unilamellar vesicles

[1] Abbreviations: αBGT, α-bungarotoxin; PBGT, palmitoyl-α-bungarotoxin; $PBGT^E$, erythrosin-labeled palmitoyl-α-bungarotoxin; $PBGT^F$, fluorescein-labeled palmitoyl-α-bungarotoxin; AchR, acetylcholine receptor; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DEPC, dielaidoylphosphatidylcholine; egg PC, egg phosphatidylcholine; FUV, fused unilamellar vesicles; GUV, giant unilamellar vesicles; MLV, multilamellar vesicles; DPH, 1,6-diphenyl-1,3,5-hexatriene; FRAP, fluorescence recovery after photobleaching; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; MOPS, 4-morpholinepropanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate.

(GUV) were prepared by a modification of the methods of Oku & MacDonald (1983). DEPC at 15 mM in 4 mL of 10 mM 4-morpholinepropanesulfonic acid (MOPS) at pH 7.2 was sonicated for 10 min at 25 °C in Heat Systems W-375 sonifer with a 0.5-in. probe. To 3 mL of the small unilamellar DEPC vesicles was added 1.5 mL of 3 M KCl followed by three cycles of freezing and thawing (30 min at room temperatur) in an acetone–dry ice bath. Finally, the turbid vesicle suspensions were placed in a Spectrapor-2 dialysis membrane and dialyzed against 4 L of 10 mM MOPS at pH 7.2 with 0.02% sodium azide for 48 h at room temperature. Vesicle size was analyzed by employing both forward and 90° laser (488-nm) light scattering (Ortho 50H flow cytometer/cell sorter) as well as negative-stain electron microscopy. The resultant DEPC GUV were approximately 2 μm in diameter with very few vesicles of diameter less than 1 μm in the population. Addition of protein at a lipid/protein molar ratio of 100 did not alter the size of the GUV.

(3) PBGT Incorporation into Phospholipid Vesicles. Eight hundred microliters of 2.87 mM DPPC FUV preequilibrated at 48 °C in 50 mM KCl containing 2.5 mM EGTA and 0.02% NaN_3 was added to a dry film of PBGT, vortexed rigorously, and incubated at 48 °C overnight to allow equilibrium incorporation of the acetylated protein into the lipid bilayer. PBGT incubated with FUV at 48 °C for much shorter periods of time failed to give reproducible calorimetric scans, indicating that stable association of the protein with the bilayer is a relativley slow process. The percent of PBGT associated with the vesicles was determined by pelleting of the FUV and counting the amount of ^{125}I-PBGT associated with the pellet. The location of the incorporated PBGT was determined by incubation of the sample with 50 units of thermolysin/mg of vesicle-associated PBGT for 30 min at 37 °C. Under these conditions 100% of the PBGT is incorporated into the lipid vesicles and essentially all of the protein is digestible with thermolysin, indicating that the protein is located on the outer surface of the vesicle. Sucrose density gradient analysis of various PBGT FUV indicated that up to a molar ratio of lipid/protein = 12 could be incorporated into these vesicles.

To incorporate $PBGT^E$ into GUV, 1 mL of 1.5 mM DEPC giant vesicles in 10 mM MOPS containing 2.5 mM EGTA and 0.02% NaN_3 was added to a dry film containing 15 nmol of $PBGT^E$, mixed gently with a Pasteur pipet, and incubated overnight at 48 °C. All of the $PBGT^E$ was incorporated on the outer surface of the GUV as measured by the thermolysin digestion mentioned above. Pelleting the giant vesicles by centrifugation or fractionation in a Bio-Gel A50m column resulted in comigration of lipid and protein, indicating that essentially all of the protein was incorporated into the phospholipid vesicles.

Incorporation of $PBGT^F$ into Multilamellar Vesicles and Measurement of Lateral Diffusion. Multilamellar vesicles of egg PC or DPPC were prepared by swelling dry phospholipids in PBS for 15 min at 60 °C, followed by rigorous vortexing. Vesicles were pelleted with a desk-top centrifuge and resuspended in PBS at approximately 10 mM in lipid. Incorporation of $PBGT^F$ into vesicles was carried out according to Cartwright et al. (1982). $PBGT^F$ (1.3 nmol in 40 μL) was added to 400 μL of the multilamellar vesicles. Concentrated deoxycholate (DOC) was added to give a final DOC concentration of 0.05%. The mixture was then dialyzed for 72 h against 6 × 2 L of PBS. The resulting liposome suspensions were centrifuged to remove small amounts of unincorporated $PBGT^F$. The vesicles were uniformly fluorescent, and the average diameter of the vesicles was approximately 5 μm as measured with epiluminescence microscopy. Lateral diffusion of $PBGT^F$ in lipid bilayers was measured by the fluorescence recovery after photobleaching (FRAP) technique in the laboratory of Dr. H. M. McConnell at Stanford University. A pattern bleaching method was used (Smith et al., 1979; Petty et al., 1980). Bleaching times of the order of 20 ms were used with a laser power density of about 10 kW/cm^2 at 488 nm. Only vesicles greater than 50 μm in diameter were measured, since the size of the bleaching grid itself was approximately 30 μm in diameter. All measurements were done at 25 °C.

High-Sensitivity Differential Scanning Calorimetry. Calorimetric data were obtained with a Microcal MC1 differential scanning calorimeter at a scanning rate of 15 °C/h. The sensitivity of the instrument has been improved by the use of two separate Keithley amplifiers connected to the heat capacity and temperature outputs of the calorimeter and interfaced to a TEC86 microcomputer system for automatic data collection and processing. The calorimetric data are digitized by a TM-AD212 A/D converter operating at a 40-kHz conversion rate and stored for subsequent analysis in floppy disks at 0.05 °C intervals. As a result pure lipid dispersions at concentrations less than 0.5 mg/mL can be used with a total sample volume of 0.7 mL. For measurements of PBGT vesicles, a concentration of 2 mg/mL lipid was used.

Steady-State Fluorescence Spectroscopy. Steady-state fluorescence depolarization measurements were performed essentially as described by Barenholz et al. (1976) using a Perkin-Elmer LS-5 fluorescence spectrophotometer equipped with a red-sensitive photomultiplier tube, a polarization accessory, and a thermostated cuvette holder connected to a Neslab RTE-8 refrigerated bath circulator equipped with a temperature programmer, ETP-3. The temperature of each sample was monitored within ± 0.1 °C by means of a Keithley digital thermometer. 1,6-Diphenyl-1,3,5-hexatriene (DPH; Molecular Probes, Junction City, OR) was dissolved in acetonitrile and added to the vesicle suspensions at a final ratio of 1 probe molecule per 500 phospholipids. Probe incorporation was allowed to continue for 1 h under a N_2 atmosphere at 45 °C prior to fluorescence measurements. Light scattering of vesicle suspensions without the probe did not exceed 2% of the fluorescence intensity perpendicular to the plane of the excitation beam. Fluorescence was measured by excitation of DPH at 360 nm, while emission was monitored at 430 nm parallel and perpendicular to the plane of excitation. Anisotropy was calculated as described elsewhere (Barenholz et al., 1976).

Time-Resolved Fluorescence and Phosphorescence Spectroscopy. The measurement of time-resolved phosphorescence depolarization was also performed with the Perkin-Elmer LS-5 fluorescence spectrophotometer. Samples were excited at 510 nm, and emission was monitored at 570 or 690 nm for delayed fluorescence or phosphorescence, respectively. In order to obtain the delayed fluorescence or phosphorescence intensity time decay, the start of the gating of the sample photomultiplier was electronically delayed from the lamp flash at increasing time intervals of 10 μs ranging from 10 to 500 μs. All liposome preparations were bubbled for 20–30 min with argon to remove O_2 prior to each measurement and the samples sealed in Teflon-stoppered cuvettes.

For anisotropy measurements the samples were excited with vertically polarized light. Time-dependent anisotropies $r(t)$ were calculated according to the formula:

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)G}{I_{\parallel}(t) + 2I_{\perp}(t)G}$$

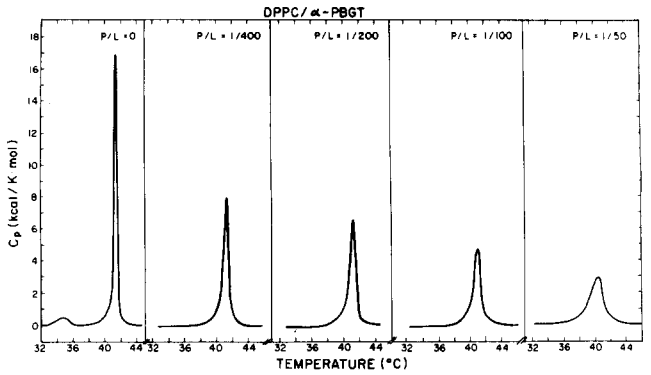

FIGURE 1: Excess heat capacity function vs. temperature for large unilamellar DPPC vesicles containing increasing molar ratios of palmitoyl-α-bungarotoxin (PBGT).

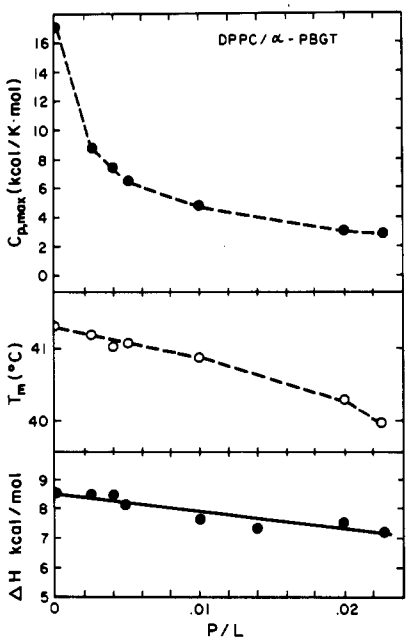

FIGURE 2: Dependence of the heat capacity maximum, $C_{p,max}$, transition temperature, T_m, and enthalpy change, ΔH, associated with the gel to liquid-crystalline transition of DPPC large unilamellar vesicles containing different molar ratios of PBGT.

where G is the correction factor obtained by using horizontally polarized light ($G = I_{\parallel}/I_{\perp}$).

Results

Scanning Calorimetry. The thermotropic behavior of DPPC FUV containing increasing mole fractions of PBGT was studied by high-sensitivity differential scanning calorimetry. As shown in Figure 1 in the absence of protein the calorimetric profile of FUV is characterized by a single sharp peak at 41.3 °C associated with the gel to liquid-crystalline transition of the phospholipid vesicles. The enthalpy change (ΔH, area under the excess heat capacity curve) for this transition is 8.6 kcal/mol, and the half-height width ($\Delta T_{1/2}$) is 0.4 °C, indicating that approximately 300 molecules of the DPPC FUV melt in a highly cooperative fashion (Freire et al., 1983). As shown in Figures 1 and 2, the addition of PBGT to these vesicles causes a monotonic decrease in both the phase transition temperature, T_m, and the enthalpy change associated with the gel to liquid-crystalline transition. Furthermore, the half-height width increases as a function of the protein mole fraction, indicating that PBGT disrupts the cooperative behavior of the lipid bilayer matrix. Repeated calorimetric scans were superimposable with initial scans, indicating that the membrane preparations were in equilibrium configuration. As shown in Figure 1, the incorporation of PBGT also affects the phospholipid pretransition, diminishing its amplitude to nondetectable levels even at the lowest mole fractions of PBGT employed. Figure 2 shows the decrease of ΔH upon increasing the PBGT/DPPC mole ratio. Such a result indicates that protein molecules are preventing some phospholipids from undergoing the lipid phase transition. Linear least-squares analysis of this dependence of ΔH on the protein/lipid mole ratio according to Correa-Freire et al. (1979) indicates that each PBGT molecule eliminates five to six phospholipids from the phase transition.

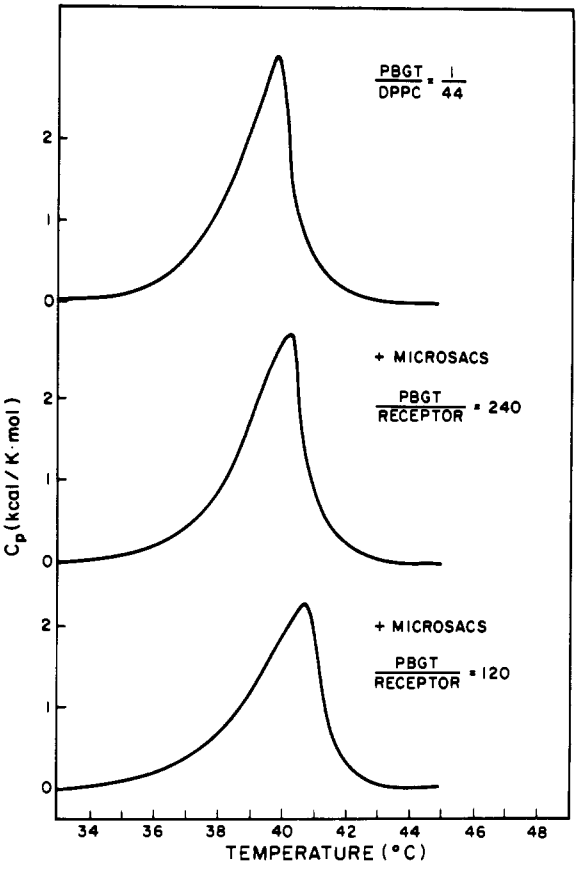

FIGURE 3: Excess heat capacity function vs. temperature for large unilamellar DPPC vesicles containing PBGT at a molar ratio of 1/44 in the absence and in the presence of microsac membranes highly enriched with the acetylcholine receptor.

The addition of plasma membranes highly enriched with the acetylcholine receptor increased slightly the transition temperature of PBGT/DPPC liposomes as shown in Figure 3. However, the enthalpy change for the transition remained constant within the experimental error, indicating that this receptor–ligand-mediated membrane–membrane interaction does not induce a major perturbation on the phase behavior of the membrane.

Fluorescence Anisotropy. In order to investigate the effect of PBGT incorporation on the fluidity of the DPPC bilayer, the steady-state emission anisotropy of PBGT/DPPC vesicles containing DPH was measured as a function of both temperature and protein mole fraction. The results of these experiments are shown in Figure 4. In agreement with the calorimetric results, a decrease in T_m dependent upon PBGT concentration was seen. In addition, the association of PBGT with the bilayer decreased the DPH anisotropy below T_m and increased the DPH anisotropy above T_m; such a result suggests that PBGT decreases the apparent order of the phospholipid acyl chains in the gel state while increasing the apparent order

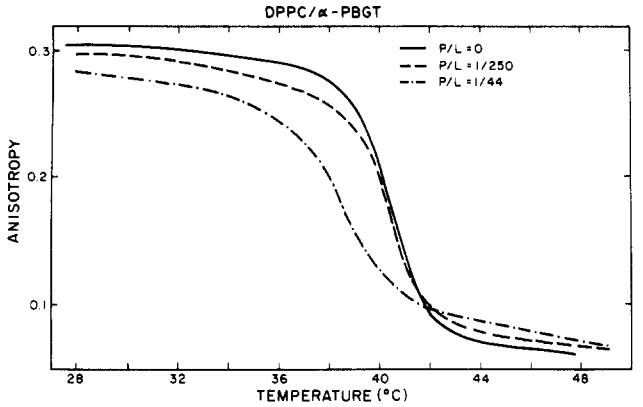

FIGURE 4: Steady-state DPH fluorescence anisotropy of large unilamellar DPPC vesicles containing no PBGT (solid line), a 1/250 PBGT/lipid molar ratio (broken line), and a 1/44 PBGT/lipid molar ratio (dotted line).

in the fluid state. This macroscopic effect of PBGT on the acyl chain packing properties of DPPC is similar to the effect observed with some intrinsic membrane proteins (Gomez-Fernandez et al., 1979).

Lateral Diffusion of PBGT^F in Lipid Bilayers. PBGT^F incorporated into multilamellar vesicles made of egg PC showed a rapid recovery of fluorescence after bleaching at 25 °C with a lateral diffusion coefficient of $(1.7 \pm 1.2) \times 10^{-8}$ cm^2/s. The fraction of fluorescence recovered was generally greater than 0.8 of the theoretical. For liposomes made of DPPC, no significant recovery of fluorescence was observed up to about 10 min after bleaching. This sets an upper limit for the lateral diffusion coefficient at 1×10^{-11} cm^2/s at 25 °C. These results indicate that lateral mobility of PBGT^F is controlled by the fluidity of the bulk lipid since, at 25 °C, the toxin molecules can diffuse rapidly in a fluid bilayer (egg PC) but not in a rigid bilayer (DPPC below T_m).

Rotational Mobility of PBGT^E in DEPC and DOPC Giant Vesicles. Contrary to fluorescein, erythrosin (tetraiodofluorescein) has a poor prompt fluorescence quantum yield but has been shown to possess a delayed emission spectrum at room temperature that can be exploited to measure the rotational dynamics of membrane proteins to which this probe has been covalently attached (Moore et al., 1979; Jovin et al., 1979). The rotational dynamics of PBGT^E incorporated into giant unilamellar DEPC and DOPC vesicles was evaluated by time-resolved delayed fluorescence emission anisotropy measurements at various temperatures. DEPC and DOPC were the lipids of choice in order to perform the experiments at or below room temperature to maximize the phosphorescence quantum yield of PBGT^E. GUV were used in order to minimize the contribution to anisotropy decay due to vesicle tumbling. This contribution might be significant for vesicles of 100-Å diameter or less (Cherry, 1979). Figure 5 shows the time-delayed emission spectrum of PBGT^E incorporated into DOPC giant vesicles at a protein/lipid molar ratio of 1/100. The spectrum was recorded at 15 °C by using an excitation wavelength of 510 nm, a delay time of 50 μs after the excitation light pulse, and a photomultiplier gating time of 50 μs. The spectrum is characterized by two peaks centered at 567 and 698 nm, respectively. The peak at 567 nm is due to delayed fluorescence emission and coincides with the prompt fluorescence peak, whereas the peak at 698 nm is due to phosphorescence emission (Moore et al., 1979). At 15 °C the phosphorescence lifetime for PBGT^E associated with either DOPC or DEPC giant unilamellar vesicles was 95 ± 4 μs whereas that for erythrosin covalently bound to bovine serum albumin (BSA^E) was 320 μs, in agreement with previous observations (Moore et al., 1979). The shorter phosphorescence lifetime of PBGT^E might be due to a different environment of the chromophore. Figure 6 shows the results of time-resolved delayed fluorescence and phosphorescence anisotropy measurements of a suspension of DOPC giant vesicles with PBGT^E incorporated at a protein/lipid molar ratio of 1/100. The concentration of PBGT^E in these experiments was 15 μM. The delayed fluorescence anisotropy values were higher than the phosphorescence anisotropy values as reported previously (Jovin et al., 1979). As shown in the figure, the delayed fluorescence anisotropy did not decay to zero but reached a constant limiting value of 0.09. Finite limiting anisotropies have been observed previously for integral membrane proteins, indicating that the motion around one of the axes of rotation is severely restricted; i.e., integral membrane proteins are presumably unable to tumble within the lipid bilayer (Cherry, 1979; Murray et al., 1983). Analysis of the decay data in

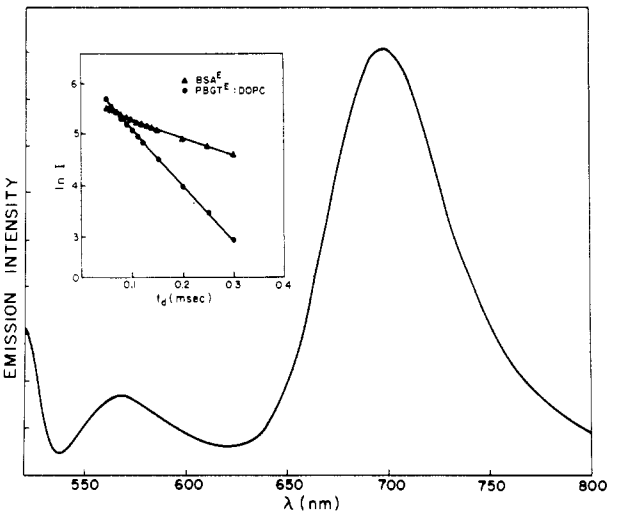

FIGURE 5: Time-delayed emission spectrum of PBGT^E incorporated into DOPC giant vesicles at a protein/lipid molar ratio of 1/100. The spectrum was recorded at 15 °C with an excitation wavelength of 510 nm, a delay time of 50 μs, and a photomultiplier gating time of 50 μs. The insert shows the natural logarithm of the phosphorescence decay vs. time for the same vesicle preparation and for erythrosin covalently bound to bovine serum albumin.

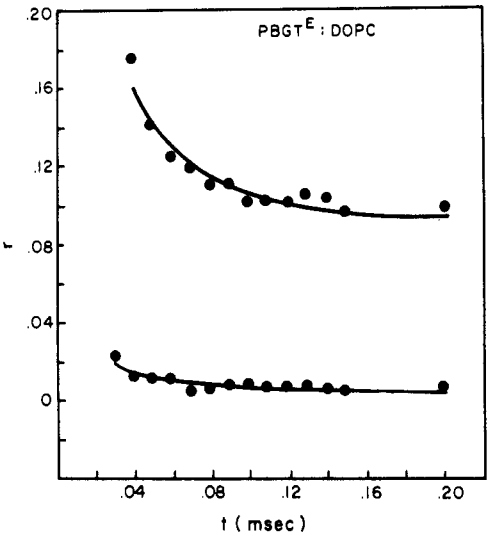

FIGURE 6: Delayed fluorescence (top curve) and phosphorescence anisotropy (bottom curve) decay curves vs. time for PBGT^E incorporated into giant DOPC vesicles. The excitation wavelength was 510 nm and the photomultiplier gating time was 50 μs. Both experiments were performed at 15 °C under oxygen-free conditions.

terms of an equation of the form

$$r(t) = A_1 + A_2 e^{-t/\phi}$$

yielded rotational correlation times (ϕ) for $PBGT^E$ of 32 ± 3 and 35 ± 4 μs by using the phosphorescence and the delay fluorescence data, respectively.

In order to assess the influence of the gel to liquid-crystalline transition on the rotational mobility of $PBGT^E$, additional experiments were performed with DEPC giant unilamellar vesicles. The phase transition temperature for this phospholipid is 11.8 °C as determined by differential scanning calorimetry (data not shown). In the liquid-crystalline phase (15 °C) the rotational correlation time was similar to the one obtained with DOPC (32 ± 2 μs). In the gel phase (9 °C), however, the rotational correlation time was 67 ± 7 μs, indicating that the rotational mobility of $PBGT^E$ was only slightly restricted upon passage of the bulk lipid from the liquid-crystalline to the gel phase. It must be noted, however, that these experiments were performed at approximately 3 °C below T_m and that it is conceivable that at much lower temperatures this phospholipid will also adopt a rigid configuration.

Discussion

The calorimetric results presented in this paper indicate that the incorporation of palmitoyl-α-bungarotoxin into DPPC FUV causes a monotonic decrease in the T_m, the enthalpy change, and the cooperativity of the main gel to liquid-crystalline phase transition in a fashion similar to that seen with a variety of integral membrane proteins (Chapman et al., 1979; Curatolo et al., 1977; Petri et al., 1980) and other components such as cholesterol (Estep et al., 1978) and glucocerebroside (Correa-Freire et al., 1979). From analysis of the dependence of ΔH on the protein to lipid molar ratio, it appears that each PBGT molecule prevents approximately five to six phospholipid molecules from participating in the main transition. This number is essentially equal to the number of phospholipids surrounding the fatty acid chain and suggests that the perturbation is of a local nature and that the acylated protein interacts with the membrane solely through the covalently attached fatty acid chain. This result is not unexpected since native α-bungarotoxin, which is highly water soluble, shows absolutely no association with dioleoylphosphatidylcholine (DOPC) vesicles as judged by sucrose density gradient centrifugation (B. Babbitt, unpublished observation). The phospholipid molecules withdrawn from the transition presumably remain in the same physical state below and above the transition temperature of the bulk lipid. Since the steady-state anisotropy measurements report a disordering effect below T_m and an ordering effect above T_m, it is conceivable that the perturbed phospholipid exists in an intermediate state between the gel and liquid-crystalline states.

The rotational correlation times of ~34 μs obtained above the gel to liquid-crystalline transition temperature are in the range expected for a low molecular weight protein anchored to the bilayer by a single fatty acid chain (see below). Immediately below T_m the rotational correlation time was reduced by about half; this is a relatively small change, indicating either that the protein has some degree of rotational freedom around the fatty acid linkage or that the immediate environment of $PBGT^E$ remains in a more or less fluidlike state even after the bulk lipid has undergone the transition to the gel state as suggested by the DPH anisotropy measurements. This latter possibility cannot be rule out since, previously, relatively small changes in protein rotational correlation times associated with the phospholipid gel to liquid crystalline have been reported for rhodopsin reconstituted into dimyristoylphosphatidylcholine vesicles (Kusumi et al., 1980) and also for the erythrocyte membrane integral protein band 3 reconstituted into dimyristoylphosphatidylcholine vesicles (Sakaki et al., 1982). Contrary to rotational correlation times, lateral diffusion coefficients were strongly sensitive to the physical state of the lipid bilayer. This is not surprising since the fluorescence recovery after photobleaching technique measures the motion of molecules along distances comparable to the linear dimensions of the membrane whereas the delayed fluorescence or phosphorescence anisotropy decay measures the rotation of molecules in their own local environment.

The diffusion of membrane components has been frequently analyzed in terms of the Saffman–Delbruck model (Saffman & Delbruck, 1975). This model assumes that the lipid bilayer is a fluid continuum of viscosity η surrounded by a liquid medium of viscosity ηw. Originally, Saffman & Delbruck (1975) derived approximate equations valid for $\eta \gg \eta w$; more recently, however, Hughes et al. (1982) reported exact calculations for all values of ηw and η. According to these authors a simultaneous measurement of the rotational and lateral mobilities can be used to estimate the membrane microviscosity and the viscosity of the surrounding medium. Assuming a length of 25 Å for the fatty acid chain attached to $PBGT^E$ and a molecular radius of 4–5 Å and using eq 1 and 2 in Hughes et al. (1982), we obtain values of 0.15–0.26 P for η and 0.03–0.08 P for ηw using the rotational and lateral diffusion coefficients obtained above the phospholipid phase transition temperature. The viscosity of water is 0.01 P, and the intrinsic viscosity of the bilayer deduced from the rotational motion of DPH in dimyristoylphosphatidylcholine bilayers (Kinosita et al., 1981) is 0.3 P. Thus, the experimental values obtained in these studies are within the range of values expected from the Saffman–Delbruck model even though the protein is anchored to the bilayer by a single fatty acid chain. Similar conclusions have been obtained before for the case of bacteriorhodopsin reconstituted in dimyristoylphosphatidylcholine vesicles (Peters & Cherry, 1982).

The association of PBGT reconstituted into phospholipid vesicles with microsac membranes highly enriched with the acetylcholine receptor induced only a small shift in the transition temperature of the phospholipid with no effect in the transition enthalpy. These results indicate that even though the toxin molecule is tightly bound to the receptor (Grant et al., 1982), this association does not induce a major change in the physical properties of the membrane. Thus, membrane–membrane association mediated by specific ligand–receptor pairs may not necessarily affect the intrinsic properties of the interacting membranes.

The results of these studies provide new insights regarding the nature of the interactions between proteins containing covalently bound fatty acids and their respective membranes. We have also demonstrated the ability to produce a membrane protein from a highly water soluble protein simply by covalent attachment of one fatty acid chain. Acylation of α-bungarotoxin, which by itself does not interact with the membrane, leads to a stable and strong association of the protein with the lipid bilayer. It is plausible that covalent attachment of a fatty acid chain to other water-soluble proteins could produce similar results. For example, it has been found that the p^{60SRC}-transforming protein of Rous sarcoma virus contains one covalently bound fatty acid chain in a region believed to participate in the association of the protein to the infected ell plasma membrane (Sefton et al., 1982). The fatty acid mediated membrane–protein association described in this paper defines a new type of protein–lipid interaction, different from

that of integral membrane proteins and peripheral membrane proteins. Even though the protein is firmly attached to the membrane, it still possesses a high degree of motional freedom absent in other membrane proteins. This enhanced mobility should influence functional responses, particularly those in which optimal reaction rates are required.

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