Lipid Phase Separations Induced by the Association of Cholera Toxin to Phospholipid Membranes Containing Ganglioside G_{M1}†

Beth Goins and Ernesto Freire*
Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37996-0840
Received August 29, 1984

ABSTRACT: The interactions of cholera toxin and their isolated binding and active subunits with phospholipid bilayers containing the toxin receptor ganglioside G_{M1} have been studied by using high-sensitivity differential scanning calorimetry and steady-state and time-resolved fluorescence and phosphorescence spectroscopy. The results of this investigation indicate that cholera toxin associates with phospholipid bilayers containing ganglioside G_{M1}, independent of the physical state of the membrane. In the absence of Ca^{2+}, calorimetric scans of intact cholera toxin bound to dipalmitoylphosphatidylcholine (DPPC) large unilamellar vesicles containing ganglioside G_{M1} result in a broadening of the lipid phase transition peak and a slight decrease (<5%) in the transition enthalpy. In the presence of Ca^{2+} concentrations sufficient to cause ganglioside phase separation, the association of the intact toxin to the membrane results in a significant decrease of enthalpy change for the lipid transition, indicating that under these conditions the toxin molecule perturbs the hydrophobic core of the bilayer. Calorimetric scans using isolated binding subunits lacking the hydrophobic toxic subunit did not exhibit a decrease in the phospholipid transition enthalpy even in the presence of Ca^{2+}, indicating that the binding subunits per se do not perturb the hydrophobic core of the bilayer. On the other hand, the hydrophobic A1 subunit by itself was able to reduce the phospholipid transition enthalpy when reconstituted into DPPC vesicles. These calorimetric observations were confirmed by fluorescence experiments using pyrene phospholipids. In these experiments, addition of Ca^{2+} ions to membrane preparations either containing intact cholera toxin or containing its binding subunit resulted in a decreased rate of excimer formation only when the hydrophobic A1 subunit of the toxin was present. These experiments indicate that penetration of the hydrophobic subunits of the toxin into the lipid bilayer matrix requires structural rearrangements of the lipid molecules and that these changes are facilitated under conditions of phase separation like the one induced by Ca^{2+} ions.

The interactions of integral membrane proteins with phospholipid bilayers have been studied by several authors using a variety of techniques. Physicochemical measurements of these interactions using spectroscopic or calorimetric techniques (Curatolo et al., 1977; Chapman et al., 1979; Marsh et al., 1982; Freire et al., 1983) indicate that integral membrane proteins induce a local perturbation of the lipid bilayer matrix and that this perturbation is limited to the layer(s) of lipid immediately adjacent to the protein. Under these conditions, overall effects arise from the superposition of local effects. The situation is different with peripheral membrane proteins in several respects. First, peripheral or surface membrane proteins interact with specific types of lipid molecules. This specificity can be of a generic type as in the case of myelin basic protein and matrix protein of vesicular stomatitis virus which bind to negatively charged phospholipids or of an absolute type like in the case of the binding subunit of cholera toxin and ganglioside G_{M1} (Fishman et al., 1978). One of the primary effects of this highly specific interaction of peripheral membrane proteins with particular lipid components of the membrane is the occurrence of lateral phase separation processes as demonstrated for the matrix protein of vesicular stomatitis virus (Wiener et al., 1983). Since peripheral proteins bind to specific types of lipid molecules, their association to the membrane surface causes a lateral redistribution of molecules giving rise to compositional asymmetries within the lipid bilayer. Those areas of the membrane in contact with the protein become enriched with a particular type of lipid while the rest of the membrane becomes devoid of it. Even though the above interactions between peripheral membrane proteins and phospholipid bilayers lead to dramatic changes in the thermotropic behavior of the membrane, these changes occur with little or no measurable effect on the enthalpy change associated with the lipid transition. Integral membrane proteins, on the other hand, always produce a significant decrease in the enthalpy change of the lipid transition, as a direct result of the perturbation of the hydrophobic core of the lipid bilayer (Curatolo et al., 1977; Chapman et al., 1979; Freire et al., 1983). Many membrane proteins contain both hydrophobic and peripheral components; until today, however, little is known about the effects of multisubunit proteins containing peripheral and integral domains on the physical properties and thermotropic behavior of the membrane. This paper addresses this question. In this paper, we describe the interactions of cholera toxin, a multisubunit protein containing both peripheral and integral domains, with phospholipid bilayer systems containing ganglioside G_{M1}. Cholera toxin is a globular protein consisting of two structurally different components (Fishman, 1982; Moss & Vaughn, 1979). The B component is made up of five identical subunits (M_r 11 000 per subunit) and binds ganglioside G_{M1} with high affinity. The A component consists of two different subunits, A1 and A2, with molecular weights of 21 000 and 5000, respectively, linked by a disulfide bond. The A component is hydrophobic and by itself cannot bind cells even though this is the toxic component able to activate adenylate cyclase. Thus, from a structural point of view, the B component can be thought of as a molecular "device" designed to facilitate the insertion of the toxic component (A component) into the

† This work was supported by grants from the National Institutes of Health (GM30819 and NS20636).

0006-2960/85/0424-1791$01.50/0

cell membrane using ganglioside G_{M1} as a receptor.

The studies presented in this paper describe the interactions between cholera toxin and their isolated integral and peripheral subunits with phospholipid bilayer vesicles containing ganglioside G_{M1} asymmetrically located on their outer layer, thus mimicking the situation found in plasma membranes. This investigation has been accomplished by combining high-sensitivity differential scanning calorimetry with steady-state and time-resolved fluorescence and phosphorescence spectroscopy. In this way, it has been possible to probe equilibrium as well as dynamic properties of the interaction and to examine the sequence of events comprising the association and subsequent penetration of cholera toxin into the membrane.

Materials and Methods

Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Biochemicals (Birmingham, AL) and used without further purification. Fused unilamellar vesicles were prepared as described elsewhere (Myers et al., 1984) essentially following the method described by Schullery et al. (1980). DPPC was N_2 dried from a chloroform solution and lyophilized overnight. The dried lipid was dissolved in 50 mM KCl containing 0.02% sodium azide to give a lipid concentration of 70 mM and vortexed rigorously at 45 °C. The white suspension was sonicated for 1 h at 45 °C by using a bath sonicator (Model G112 SP1G, Laboratory Supplies, Hicksville, NY). The suspension was then centrifuged at 15000*g* for 1 h above the phase transition temperature. The small sonicated vesicles found in the upper two-thirds of the supernatant were incubated at 4 °C for at least 1 week as described by Wong et al. (1982). This low-temperature incubation produces a homogeneous population of fused unilamellar vesicles with a diameter of 900 Å (Wong et al., 1982).

Giant unilamellar vesicles of 1-μm diameter were prepared by a modification of the rapid freeze–thaw and dialysis procedure as described by Oku & MacDonald (1983). Three milliliters of 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.2) was added to a dried film of lipid to give a final lipid concentration of 15 mM. Small sonicated vesicles were formed as previously described in the formation of fused unilamellar vesicles. One milliliter of 3 M KCl was added to the upper two-thirds supernatant (~2 mL of small sonicated vesicles), and the mixture was subjected to 3 cycles of freeze–thaw. The sample was frozen for 2 min in dry ice–acetone and allowed to thaw at room temperature (30 min). Then the vesicles were dialyzed for 2 days in 10 mM MOPS buffer containing 0.02% sodium azide.

Ganglioside G_{M1} from beef brain was a generous gift from Dr. T. E. Thompson (University of Virginia). The fatty acid composition of beef brain gangliosides is primarily $C_{18:0}$ (>80%), the rest being primarily a mixture of $C_{20:0}$, $C_{22:0}$, and $C_{24:0}$ chains (Svennerholm, 1964; Sillerud et al., 1979). Incorporation of the ganglioside into the fused unilamellar vesicles was performed by adding a given amount of stock solution of G_{M1} in 50 mM KCl and incubating the mixture above the phase transition temperature for 90 min. This allows for an asymmetric distribution of ganglioside in which ganglioside is found only on the outer surface of the vesicles (Felgner et al., 1981). Except when otherwise indicated, ganglioside concentration in all experiments was 10 mol % of the total lipid concentration.

Lipid concentrations were estimated by a modified Bartlett phosphate assay as described by Marinetti (1962). Ganglioside concentrations were calculated from total sialic acid determinations by using a resorcinol method similar to that described by Spiro (1966). In this assay, samples and standards in 0.5-mL aqueous solutions are incubated with 0.5 mL of resorcinol solution (2 mL of 2% resorcinol, 16 mL of concentrated HCl, 0.05 mL of 0.1 M $CuSO_4 \cdot 5H_2O$, and 2 mL of H_2O) for 15 min in a boiling water bath. After the mixture cooled, 2 mL of 1-butanol is added, the samples are mixed thoroughly, and the absorbance at 585 nm is determined. Standards of 3–30 μg of sialic acid from a 0.1 M stock are used.

Intact cholera toxin was purchased from Sigma (St. Louis, MO). Isolated cholera toxin B subunits were obtained from Sigma and Calbiochem (La Jolla, CA). The proteins were supplied as lyophilized powders and were reconstituted with water to give 1 mg/mL protein in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, 0.2 M NaCl, 3 mM NaN_3, and 1 mM disodium ethylenediaminetetraacetic acid (Na_2EDTA), pH 7.5. The A1 portion was cleaved from cholera toxin by treating the toxin with a 5-fold molar excess of mercaptoethanol and incubating overnight at 37 °C (Gill, 1977; Dwyer & Bloomfield, 1982). This hydrophobic fragment forms a white precipitate which can easily be separated from $A2B_5$ by centrifugation at 15000*g* for 30 min. The A1 pellet was washed once with buffer. The supernatant containing the $A2B_5$ complex was dialyzed against Tris overnight at room temperature to remove the mercaptoethanol. Protein concentrations were checked by the method of Lowry (1951).

1-Palmitoyl-2-pyrenedecanoylphosphatidylcholine (pyrene-PC) was obtained from KSV-Chemicals (Helsinki, Finland). A stock solution of the probe was prepared by using absolute ethanol as the solvent and stored at −20 °C in the dark under argon.

Covalent Labeling of Cholera Toxin. A stock solution of erythrosin isothiocyanate (Molecular Probes) was prepared in acetone and stored at −20 °C in the dark. The intact cholera toxin was reconstituted as described previously except that the final sample pH was 8.5. The protein was added to a dried film of erythrosin isothiocyanate at a 1.2 molar excess of label and incubated for 2 h at room temperature. Excess label was removed by a rapid gel filtration–centrifugation technique (Tuszynski, 1980). With an extinction coefficient of 83 mM^{-1} cm^{-1} at 540 nm for erythrosin isothiocyanate, the final reaction product was found to contain one probe molecule covalently attached for each protein molecule.

Differential Scanning Calorimetry. Calorimetric experiments were performed by using a Microcal MCI differential scanning calorimeter operating at a scanning rate of 14.3 °C/h. The sensitivity and precision of the basic calorimetric unit have been improved by using a separate Keithley amplifier connected to the heat capacity output of the instrument and interfacing the calorimetric unit to an IBM PC microcomputer for automatic data collection and processing. The calorimetric data are digitized by an AD converter board (Data Translation DT-805) and stored in floppy disks at 0.05 °C intervals for subsequent analysis. Lipid concentrations for all experiments were 3 μmol/mL. In the experiments using the intact toxin and B subunit, DPPC-fused unilamellar vesicles containing 10% G_{M1} were added to stock solutions of the proteins. The A1 subunit was reconstituted into DPPC-fused vesicles by using a method similar to that described by Babbitt et al. (1984). The A1 subunit was vigorously vortexed with a vesicle suspension at 42 °C and incubated overnight at the same temperature to allow incorporation into the membrane.

Fluorescence Spectroscopy. All fluorescence experiments were performed in a Perkin-Elmer LS-5 spectrofluorometer equipped with a red-sensitive photomultiplier, a polarization accessory, and a thermostated cuvette holder connected to a

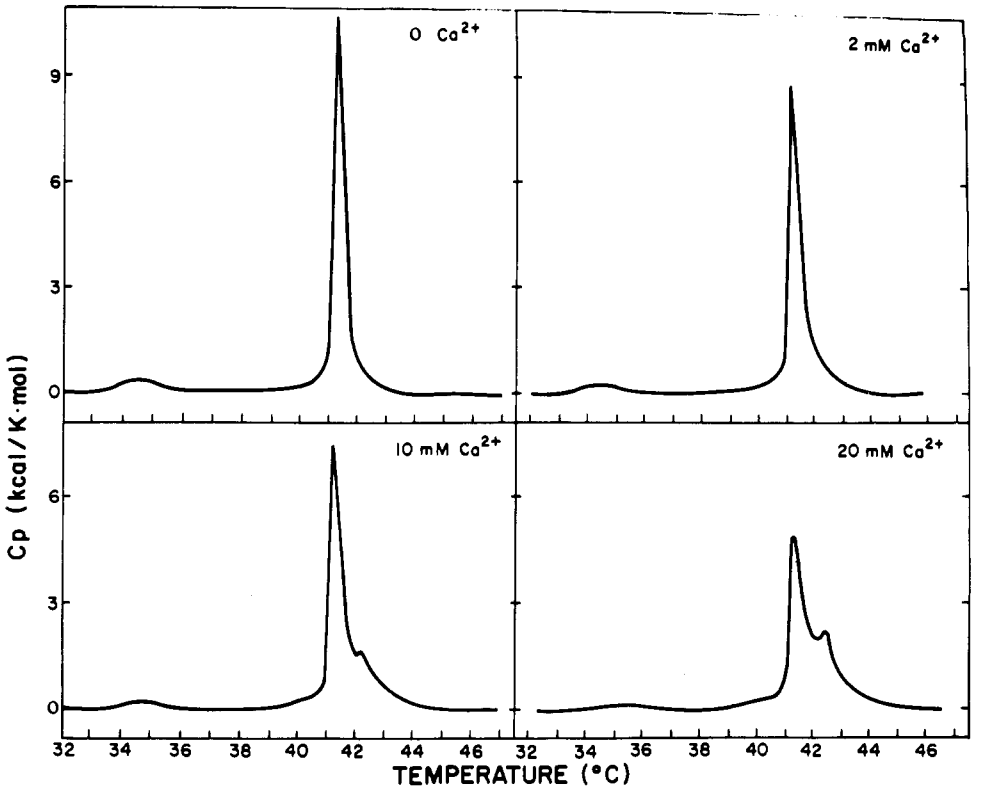

FIGURE 1: Heat capacity function vs. temperature at different Ca^{2+} concentrations for DPPC large unilamellar vesicles containing 10% ganglioside G_{M1}.

Precision RDL 20 bath circulator. The temperature of the samples was monitored during the experiments with a Keithley digital thermometer. Excimer formation experiments were performed by measuring the fluorescence emission spectrum of pyrene-PC incorporated into vesicles containing 10% G_{M1} at increasing concentrations of protein. In these experiments, the samples were excited at 320 nm, and the emissions of monomer and dimer were taken at their maxima at 395 and 480 nm.

Phosphorescence Anisotropy Measurements. The Perkin-Elmer LS-5 spectrofluorometer was also employed in the time-resolved fluorescence and phosphorescence depolarization experiments. The samples were excited at 510 nm, and the emission was monitored at 570 or 690 nm for delayed fluorescence or phosphorescence, respectively. 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. Prior to each measurement, the samples were bubbled for 30 min with argon to remove the O_2 and sealed in Teflon-stoppered cuvettes. Time-dependent anisotropies, $r(t)$, were calculated by using the following formula:

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

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

Results

Tryptophan Fluorescence of Cholera Toxin. The ability and specificity of cholera toxin to bind phospholipid vesicles containing ganglioside G_{M1} were checked by observing the tryptophan emission spectrum of cholera toxin in solution and after the addition of vesicle preparations of various molecular compositions. In aqueous solution or after addition of phosphatidylcholine vesicles or vesicles containing ganglioside G_{t1b}, the fluorescence emission spectrum remained unchanged with a maximum at 348 nm (excitation wavelength = 280 nm). Addition of vesicles containing the toxin receptor ganglioside G_{M1}, however, causes an instantaneous blue shift in the fluorescence spectrum, indicating that the tryptophan residues of the protein become exposed to a less polar environment (Fishman et al., 1978). This shift in the fluorescence emission spectrum after addition of DPPC–G_{M1} vesicles occurred both below and above the phospholipid phase transition temperature, indicating that cholera toxin is able to bind ganglioside G_{M1} independently of the physical state of the lipid bilayer.

Thermotropic Behavior of DPPC–G_{M1} Cholera Toxin Vesicles. The incorporation of up to 10 mol % ganglioside G_{M1} into large unilamellar DPPC vesicles has only a small effect on the thermotropic behavior of the vesicles. The main gel–liquid-crystalline transition of these vesicles is characterized by an enthalpy change of 8.5 kcal/mol, a transition temperature of 41.3 °C, and a half-height width of 0.4 °C. The enthalpy change for the transition remains unchanged in the presence of up to 10 mol % ganglioside G_{M1}, but the heat capacity peak becomes broader and skewed toward the high-temperature end of the transition as illustrated in Figure 1. These results agree with those obtained previously by Sillerud et al. (1979). The presence of Ca^{2+} in concentrations as low as 2 mM further accentuates the skewness of the peak, and at higher concentrations, a second calorimetric peak centered at 42.5 °C becomes clearly discernible in the calorimetric scans. The total area under the curve remains constant and independent of Ca^{2+} concentration within experimental error. Under the conditions of these experiments, the area under the second peak is not greater than 15% of the total area even under excess Ca^{2+}, suggesting that Ca^{2+} primarily phase separates the negatively charged ganglioside G_{M1} molecules. Previously (Myers et al., 1984), we have reported a qualitatively similar pattern for the trisialoganglioside G_{t1b}.

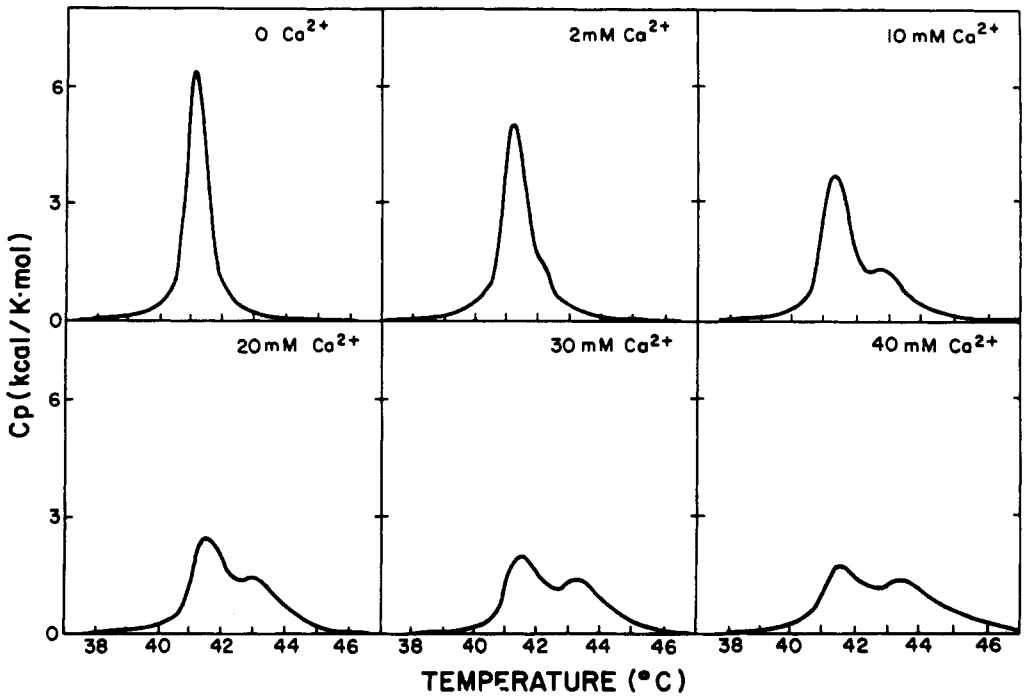

FIGURE 2: Heat capacity function vs. temperature at different Ca^{2+} concentrations for DPPC large unilamellar vesicles containing 10% ganglioside G_{M1} in the presence of cholera toxin. The protein to lipid ratio was 1/210.

The calorimetric results for DPPC–G_{M1} vesicles in the presence of intact cholera toxin are shown in Figure 2. The addition of cholera toxin at a protein/lipid ratio of 1/210 to a vesicle preparation containing 10 mol % ganglioside G_{M1} results in a broadening of the calorimetric peak and a small decrease of 0.2 °C in the transition temperature. The enthalpy change of the lipid transition is only slightly diminished (<5%), indicating that the hydrophobic core of the bilayer is not perturbed significantly. In the presence of Ca^{2+}, however, the effect of cholera toxin is more pronounced. Even at concentrations as low as 2 mM Ca^{2+}, the presence of a second peak is clearly distinguishable. At higher Ca^{2+} concentrations, the second high-temperature peak is shifted upward and becomes very prominent, occupying approximately 40% of the total area. It should be noted that the enthalpy change for the phospholipid transition decreases upon increasing Ca^{2+} concentration. This decrease in enthalpy upon increasing Ca^{2+} concentration is absent in the absence of cholera toxin and is indicative of a larger perturbation of the hydrophobic core of the membrane by the toxin molecule. The decrease in ΔH observed in the presence of Ca^{2+} is reminiscent of that observed with integral membrane proteins (Curatolo et al., 1977; Chapman et al., 1979; Freire et al., 1983). Analysis of this decrease in enthalpy as described previously (Correa-Freire et al., 1979; Freire et al., 1983) indicates that each cholera toxin prevents approximately 35 phospholipids from participating in the gel–liquid-crystalline transition. This number is consistent with calorimetric results obtained for a variety of integral membrane proteins and with the molecular dimensions of the hydrophobic A subunit reported by Dwyer & Bloomfield (1982). Thus, in the presence of Ca^{2+}, the effect of cholera toxin on the thermotropic behavior of the membrane appears to be 2-fold: (1) a rather large phase separation process involving not only ganglioside G_{M1} but also a significant fraction of the neutral phospholipid; and (2) a decrease in ΔH similar to that observed for integral membrane proteins.

Effects of Cholera Toxin Subunits on Thermotropic Behavior of DPPC–G_{M1} Vesicles. To evaluate the individual roles played by the binding (B_5) and active (A1) subunits of cholera toxin on the overall membrane interaction, calorimetric experiments were performed with membrane preparations to which only the A or B subunits of the toxin had been added. The B_5 subunit complex had a similar effect to that of the intact cholera toxin on the thermotropic behavior of the DPPC–G_{M1} vesicles, the only difference being that in the case of the B_5 subunit complex the enthalpy change of the lipid transition remained constant even in the presence of Ca^{2+}. On the contrary, the hydrophobic A1 subunit displayed an effect similar to that found for integral membrane proteins; i.e., it lowered the transition temperature and reduced the enthalpy change associated with the phospholipid gel–liquid-crystalline transition (Curatolo et al., 1977; Chapman et al., 1979; Freire et al., 1983) without causing the appearance of two phase-separated peaks. It must be noted that the A1 subunit did not require ganglioside G_{M1} in order to have a perturbing effect on the membrane, in agreement with previous observations that only the B subunit interacts specifically with ganglioside G_{M1}. The calorimetric results suggest that the overall membrane perturbation by a complex multisubunit protein containing a peripheral and a hydrophobic region is an additive process and that only those regions of the protein in direct contact with the hydrophobic core of the bilayer are able to induce a decrease in the enthalpy change of the lipid transition. Studies with the B_5 subunit complex of cholera toxin and other peripheral membrane proteins like the matrix protein of vesicular stomatitis virus (Wiener et al., 1983) indicate that surface proteins are able to induce rather large phase separation processes within the membrane without an appreciable effect on the enthalpy change for the lipid transition.

Cholera Toxin Effect on Lipid Lateral Mobility. The rate of excimer formation by pyrene phospholipid derivatives is a diffusion-controlled process and has been widely used to estimate lipid lateral mobilities within the bilayer matrix [for a review, see Galla & Hartman (1980)]. In our experiments, the ratios I'/I of the fluorescence intensity of pyrene-PC at the maxima of the excimer (I') and monomer (I) bands were measured as a function of the toxin or toxin B_5 subunit concentration. All these experiments were performed at 45 °C,

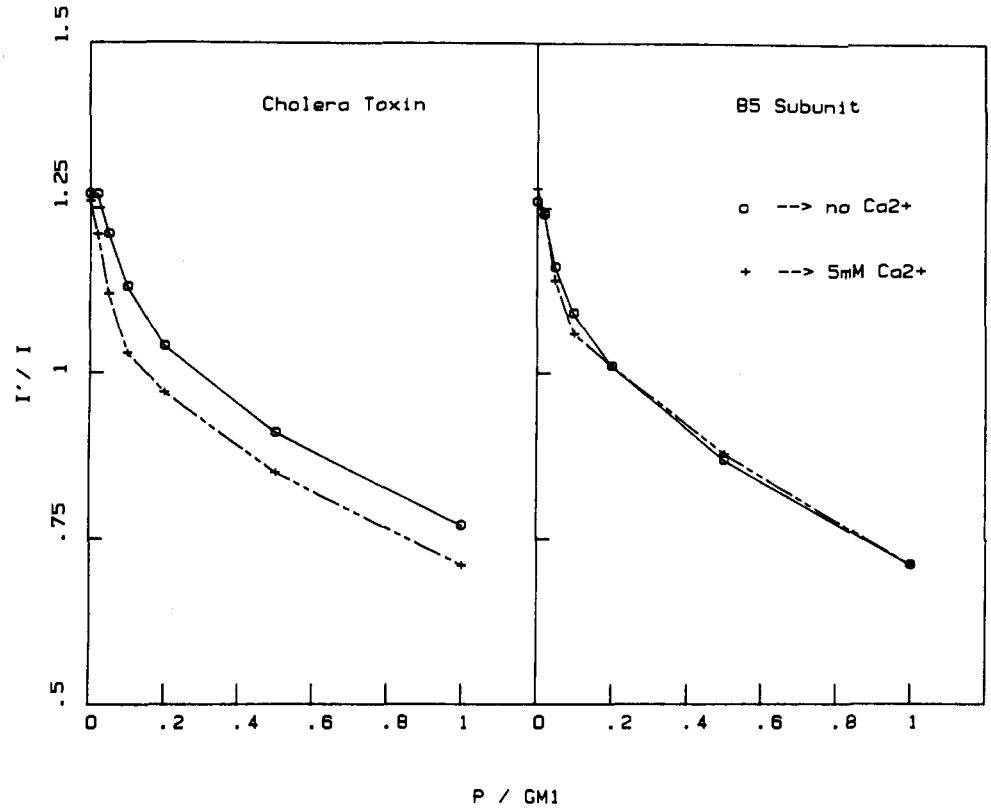

FIGURE 3: Excimer/monomer ratios of pyrenephosphatidylcholine incorporated into large unilamellar DPPC vesicles containing 10% G_{M1} vs. increasing concentrations of intact cholera toxin or B subunit in the presence or absence of 5 mM Ca^{2+}. The samples were excited at 320 nm, and the emissions of monomer and dimer were taken at their maxima of 395 and 480 nm, respectively. The lipid concentration was 3 μM, and the amount of pyrenephosphatidylcholine was 5 mol % of the total lipid.

i.e., above the lipid phase transition temperature. As shown in Figure 3, the addition of increasing amounts of cholera toxin or the B_5 subunit complex caused a decrease in the rate of excimer formation consistent with a reduced lateral mobility of the phospholipid probe. For the membrane preparations containing intact toxin, the rates of excimer formation were sensitive to the presence of Ca^{2+} ions. At all toxin concentrations studied, the excimer/monomer ratios were smaller in the presence than in the absence of Ca^{2+} as indicated in Figure 3. On the contrary, the rates of excimer formation were rather insensitive to the presence of Ca^{2+} ions when only the B_5 subunit complex was added to the membrane preparations. These experiments are consistent with the calorimetric results and indicate that the association of the intact toxin or the B_5 subunit complex to the membrane surface results in a reduced lateral mobility of the phospholipid molecules within the bilayer. For the intact toxin, a further decrease in lateral mobility was observed in the presence of Ca^{2+}, in agreement with the calorimetric observation that under these conditions cholera toxin perturbs the hydrophobic core of the bilayer in a more pronounced way, probably due to a deeper penetration of the A subunit into the hydrophobic core of the bilayer.

Rotational Mobility of Cholera Toxin. It has been shown previously (Moore et al., 1979; Murray et al., 1983; Babbit et al., 1984) that eosin and erythrosin chromophores exhibit delayed fluorescence and phosphorescence emission at room temperature and that this delayed emission can be used to evaluate the rotational mobility of membrane-associated proteins. For our studies, cholera toxin was covalently labeled at a 1/1 molar ratio with erythrosin isothiocyanate. Figure 4 shows the delayed emission spectrum of erythrosin-labeled cholera toxin at 20 °C. The spectrum was measured by delaying the photomultiplier gating time 50 μs after the excitation light pulse. As shown in the figure, the delayed emission spectrum is characterized by two maxima, one at 570 nm corresponding to the delayed fluorescence emission and one at 690 nm corresponding to the phosphorescence emission. At 20 °C, the phosphorescence lifetime (insert in Figure 4) of erythrosin-labeled cholera toxin is 250 μs, i.e., somewhat shorter than the lifetime of 320 μs obtained by us (Babbit et al., 1984) and other groups (Moore et al., 1979) for erythrosin covalently bound to bovine serum albumin under identical conditions. These differences most probably reflect the different chemical environment of the chromophore. In solution, the delayed fluorescence and the phosphorescence anisotropies of the erythrosin-labeled toxin were zero, as expected for an 83 000-dalton protein in the microsecond time scale. After addition of giant DPPC vesicles containing G_{M1}, the delayed fluorescence anisotropy increased dramatically to 0.2 and the phosphorescence anisotropy to 0.1. Both anisotropies decayed with a rotational correlation time in the millisecond time scale, indicating that in the gel phase the toxin molecule becomes strongly immobilized upon binding to the membrane.

The effect of temperature on the rotational mobility of cholera toxin associated to DPPC giant unilamellar vesicles containing ganglioside G_{M1} was examined by using time-averaged phosphorescence anisotropy as proposed by Murray et al. (1983). For these experiments, the phosphorescence emission parallel and perpendicular to the plane of polarization of the excitation pulse was integrated from 0.05 to 8 ms and the anisotropy function calculated as discussed above. Figure 5 shows the result of such an experiment. Below the transition temperature, the time-averaged anisotropy ranged between 0.08 and 0.09, indicating that the cholera toxin molecule is almost totally immobilized on the membrane surface. Previously, Murray et al. (1983) have obtained time-averaged phosphorescence anisotropy values of 0.08 for erythrosin-labeled sarcoplasmic reticulum ATPase immobilized by cross-

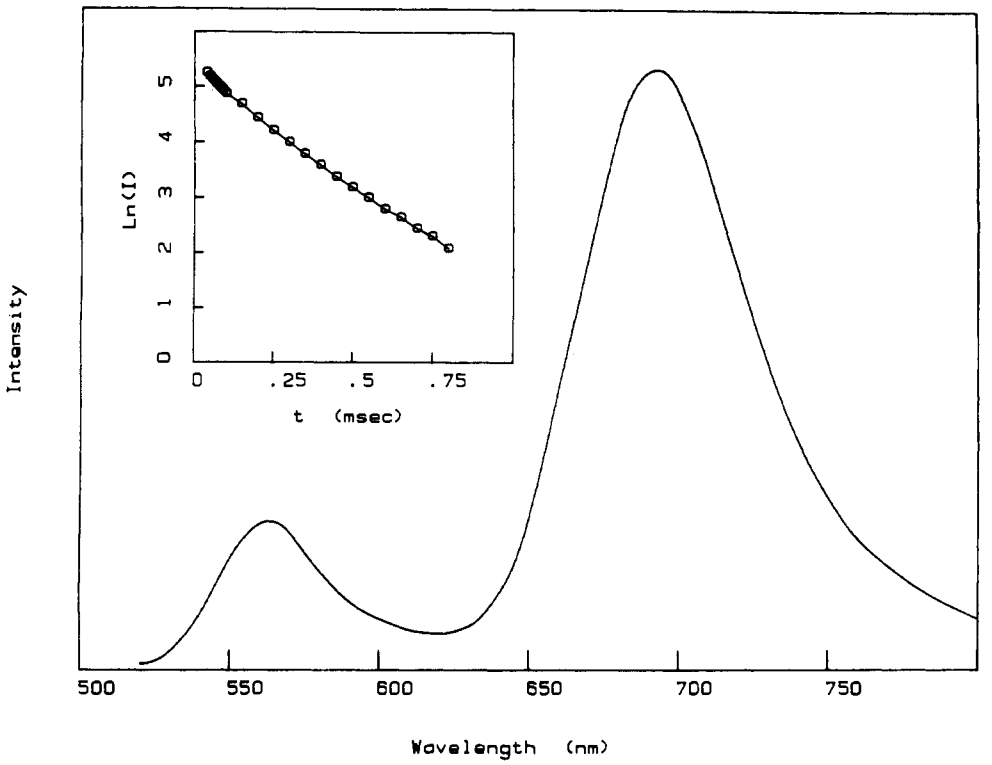

FIGURE 4: Phosphorescence emission spectrum of erythrosin isothiocyanate labeled cholera toxin at 20 °C. The excitation wavelength was 510 nm. Inset: Natural logarithm of the phosphorescence intensity (690 nm) vs. decay time for the labeled cholera toxin. The lifetime of the probe was 0.25 ms.

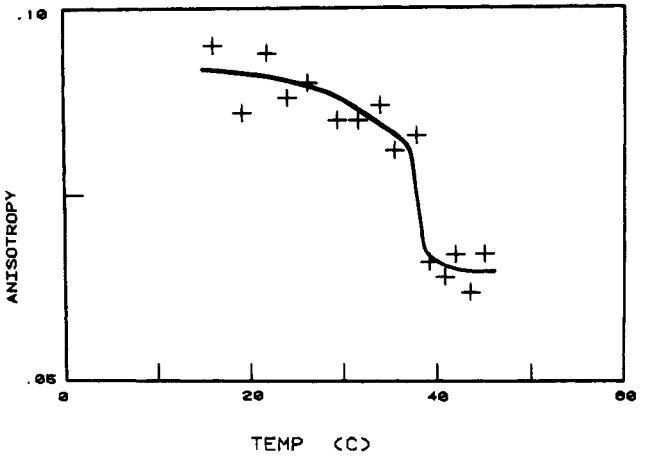

FIGURE 5: Time-averaged phosphorescence anisotropy vs. temperature for erythrosin isothiocyanate labeled cholera toxin in the presence of DPPC giant unilamellar vesicles containing 10% ganglioside G_{M1}. The phosphorescence anisotropy was integrated from $T_0 = 0.05$ ms to $T_f = 8$ ms after the light pulse.

linking following incubation with glutaraldehyde. At the lipid phase transition temperature, the time-averaged phosphorescence anisotropy decreases rather abruptly to a value of 0.07. This is still a relatively high value (Murray et al., 1983), suggesting that the rotational motion of the cholera toxin molecule is still somewhat restricted above the lipid phase transition temperature. The transition temperature measured by phosphorescence anisotropy of the labeled cholera toxin was ~1 °C lower than that measured by calorimetry. This difference may be an indication that, in the absence of Ca^{2+}, the protein-bound lipid domains have a slightly lower transition temperature than the bulk lipid.

DISCUSSION

The interaction of cholera toxin with lipid bilayer membranes is initiated by the specific association of the toxin molecules to ganglioside G_{M1}. This initial association is independent of the physical state of the lipid bilayer as indicated by the observed changes in the toxin fluorescence spectrum and by rotational mobility measurements of erythrosin isothiocyanate labeled toxin in solution and after the addition of DPPC–G_{M1} vesicles below the lipid phase transition temperature. The second event in the cholera toxin–membrane interaction is the penetration of the active subunit into the hydrophobic core of the membrane. This event consists of at least two separate processes: (1) a conformational change in the toxin molecule itself that exposes the hydrophobic active subunit to the membrane surface [evidence for a conformational change of the toxin molecule upon binding to ganglioside G_{M1} has been obtained from circular dichroism experiments by Fishman et al. (1978)]; (2) insertion of the active subunit into the hydrophobic core of the bilayer. This step is expected to require some changes in the lipid structural organization in order to permit the accommodation of a 21 000-dalton protein within the bilayer. Tomasi & Montecucco (1981) concluded on the basis of photolabelling experiments using photoreactive lipids that reduction of the A1–A2 disulfide bridge was a prerequisite for protein insertion and that in the absence of a reducing agent the A1 subunit was facing the aqueous environment and not the membrane surface. More recent studies (Dwyer & Bloomfield, 1982) using quasi-elastic laser light scattering are consistent with a model in which the A1 subunit faces the membrane surface upon binding of the toxin to the ganglioside and that bilayer penetration or at least partial penetration by the A1 subunit does not require the presence of a reducing agent. This latter model seems more plausible from a thermodynamic point of view in the sense that the hydrophobic A1 subunit never becomes exposed to the aqueous environment.

The calorimetric results presented in this paper indicate that the binding of the intact cholera toxin molecule to DPPC vesicles containing ganglioside G_{M1} does not have any large

effect on the thermotropic behavior of the lipid vesicles. The effect is mainly restricted to a broadening of the heat capacity peak and a very small (<5%) decrease in the enthalpy change for the transition. The small decrease in ΔH indicates that the toxin molecule is not significantly perturbing the hydrophobic core of the bilayer. Under the same conditions, a hydrophobic protein with the same molecular dimensions as the A1 subunit of cholera toxin would be expected to decrease ΔH by 20%. In the presence of Ca^{2+}, however, we observed a decrease in ΔH indicative of a larger perturbation of the hydrophobic core of the bilayer. This decrease in ΔH is of the expected magnitude for the insertion of the A1 subunit. It is important to mention that this decrease in ΔH is not observed in the experiments performed with the B_5 subunit complex lacking the A1 subunit and that the A1 subunit alone produces a similar decrease in ΔH after reconstitution with the lipid vesicles. Thus, the calorimetric results are consistent with the model presented by Dwyer & Bloomfield (1982) regarding the orientation of the A1 subunit toward the membrane surface and furthermore indicate that Ca^{2+} facilitates the insertion of the hydrophobic subunit into the membrane. Different authors (Swaney, 1980; Pownall et al., 1981) have shown that the insertion of proteins into lipid bilayers is facilitated by phase separation processes. In the present case, Ca^{2+} promotes phase separation of ganglioside G_{M1} as demonstrated by the calorimetric scans in Figure 2, and under these circumstances, the hydrophobic components of the toxin molecule are apparently able to penetrate into the bilayer matrix.

The fluorescence experiments using pyrene-labeled phosphatidylcholine support the results obtained by differential scanning calorimetry. Addition of cholera toxin produces a systematic decrease in the rate of excimer formation consistent with a reduced lateral mobility of the lipid molecules. Fluorescence photobleaching recovery experiments (B. Goins, B. G. Barisas, and E. Freire, unpublished results) also report a similar decrease in the ganglioside lateral diffusion coefficient upon addition of cholera toxin to dimyristoylphosphatidylcholine (DMPC) vesicles containing ganglioside G_{M1}. A similar decrease in the rate of excimer formation was obtained after addition of the B_5 binding subunit, suggesting, as in the case of the calorimetric experiments, that in the absence of Ca^{2+} there is no significant bilayer perturbation induced by the presence of the active A1 subunit. In the presence of Ca^{2+}, however, the situation is different. For the intact toxin, a further decrease in the rate of excimer formation was observed whereas none was observed for the B_5 subunit. This is again consistent with the idea that Ca^{2+} allows a deeper penetration of the A1 subunit into the hydrophobic core of the bilayer.

The picture that emerges from the above experiments is that cholera toxin binds ganglioside G_{M1} on the membrane surface independent of the physical state of the bilayer. Upon binding to G_{M1}, the A1 subunit faces the membrane surface as suggested by Dwyer & Bloomfield (1982); however, the A1 subunit does not significantly penetrate into the hydrophobic core of the bilayer. Presumably, insertion of the A1 subunit requires disruption of the packing arrangement of the lipid molecules, and this event can be triggered or accelerated by phase separation processes like the one induced by Ca^{2+} ions.

Registry No. Ganglioside G_{M1}, 37758-47-7.

REFERENCES

Babbitt, B., Huang, L., & Freire, E. (1984) *Biochemistry 23*, 3907–3912.

Chapman, D., Gomez-Fernandez, J. C., & Goni, F. M. (1979) *FEBS Lett. 98*, 211–228.

Correa-Freire, M. C., Freire, E., Barenholz, Y., Biltonen, R., & Thompson, T. E. (1979) *Biochemistry 18*, 442–445.

Curatolo, W., Sakura, J. D., Small, D. N., & Shipley, G. G. (1977) *Biochemistry 16*, 2313–2319.

Dwyer, J. D., & Bloomfield, V. A. (1982) *Biochemistry 21*, 3227–3231.

Felgner, P. L., Freire, E., Barenholz, T., & Thompson, T. E. (1981) *Biochemistry 20*, 2168–2172.

Fishman, P. H. (1982) *J. Membr. Biol. 69*, 85–97.

Fishman, P. H., Moss, J., & Osborne, J. C. (1978) *Biochemistry 17*, 711–716.

Freire, E., Markello, T., Rigell, C., & Holloway, P. W. (1983) *Biochemistry 22*, 1675–1680.

Galla, H. J., & Hartmann, E. (1980) *Chem. Phys. Lipids 27*, 199–219.

Gill, D. M. (1977) *Adv. Cyclic Nucleotide Res. 8*, 85–118.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marinetti, G. V. (1962) *J. Lipid Res. 3*, 1–20.

Marsh, D., Watts, A., Pates, R. D., Uhl, R., Knowles, P. F., & Esmann, M. (1982) *Biophys. J. 37*, 265–271.

Moore, C., Boxer, D., & Garland, P. (1979) *FEBS Lett. 108*, 161–166.

Moss, J., & Vaughn, M. (1979) *Annu. Rev. Biochem. 48*, 560–581.

Murray, E. K., Restall, C. J., & Chapman, D. (1983) *Biochim. Biophys. Acta 732*, 347–351.

Myers, M., Wortman, C., & Freire, E. (1984) *Biochemistry 23*, 1442–1448.

Oku, N., & MacDonald, R. C. (1983) *Biochemistry 22*, 855–863.

Pownall, J. H., Pao, Q., Hickson, D., Sparrow, J. T., Kusserow, S. K., & Massey, J. B. (1981) *Biochemistry 20*, 6630–6635.

Schullery, S. E., Schmidt, C. F., Felgner, P., Tillack, T. W., & Thompson, T. E. (1980) *Biochemistry 19*, 3919–3923.

Sillerud, L. O., Schafer, D. E., Yu, R. K., & Konigsberg, W. H. (1979) *J. Biol. Chem. 21*, 10876–10880.

Spiro, R. G. (1966) *Methods Enzymol. 8*, 3–16.

Svennerholm, L. (1964) *J. Lipid Res. 5*, 145–155.

Swaney, J. B. (1980) *J. Biol. Chem. 255*, 8791–8796.

Tomasi, M., & Montecucco, E. (1981) *J. Biol. Chem. 256*, 11177–11181.

Tuszynski, G. P., Knight, L., Piperno, J. R., & Walsh, P. N. (1980) *Anal. Biochem. 106*, 118–122.

Wiener, J., Wagner, R. R., & Freire, E. (1983) *Biochemistry 22*, 6117–6123.

Wong, M., Anthony, F. H., Tillack, T. W., & Thompson, T. E. (1982) *Biochemistry 21*, 4126–4132.