To the Graduate Council:

I am submitting herewith a dissertation written by Beth Ann Goins entitled "Calorimetric and Fluorescent Characterization of the Interactions of Cholera Toxin with its Cell Surface Receptor, Ganglioside G_{M1}." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

Ernesto Freire

Ernesto Freire, Major Professor

We have read this dissertation
and recommend its acceptance:

Leaf Huang

John Koontz

Jeffrey M Boss

Accepted for the Council:

Lee Minkel

Vice Provost
and Dean of The Graduate School

CALORIMETRIC AND FLUORESCENT CHARACTERIZATION OF THE INTERACTIONS OF CHOLERA TOXIN WITH ITS CELL SURFACE RECEPTOR, GANGLIOSIDE G_{M1}

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Beth Ann Goins

June 1988

ACKNOWLEDGMENTS

I gratefully acknowledge Dr. Ernesto Freire for his encouragement and guidance. Without his scientific knowledge and insight, this research project would have been difficult to complete.

I would like to thank Dr. John Koontz, Dr. Leaf Huang and Dr. Jeff Becker for giving of their valuable time to serve on my thesis committee.

Many thanks to Dr. George Barisas and Dr. Deborah Roess who taught me the technique of fluorescence photobleaching recovery and made my stay at Colorado State University a pleasant one. I am also grateful to Dr. Richard Haugland of Molecular Probes who suggested using CMTAA-eosin for the ganglioside-labeling studies and to Dr. T. E. Thompson for providing me with ganglioside G_{M1}.

I would like to thank my fellow students and colleagues in the lab, especially Mrs. Cora Wortman who supplied me with gangliosides for the ganglioside G_{M1} purification. Also I am grateful to Ms. Anne Upchurch for her excellent typing of this thesis.

I would especially like to thank my parents, Rex and Geneva Goins, for their encouragement and financial support during my graduate education.

ABSTRACT

The physical nature of the sequence of events associated with the binding and insertion of cholera toxin into membranes was studied using high sensitivity differential scanning calorimetry (DSC), fluorescence spectroscopy, isothermal reaction calorimetry and differential solubility thermal gel analysis. This sequence of events consists of 1) association of toxin to membranes containing ganglioside G_{M1}, the toxin cell surface receptor and 2) release and penetration of the toxin bioactive (A) subunit into receptor-containing membranes.

The effect of ganglioside G_{M1} association on toxin structure and function was studied by monitoring toxin thermal stability using DSC and differential solubility thermal gel analysis. The results of this investigation showed 1) stabilization of the B subunit following ganglioside binding 2) enhancement of the cooperative interaction between B monomers in the B pentamer and 3) absence of an exothermic heat effect due to A subunit precipitation.

The interactions of toxin with phospholipid-G_{M1} bilayers indicate the toxin associates to both phases of the bilayer. Also toxin association results in only a small decrease in the ganglioside lateral mobility. DSC studies of intact toxin or its isolated subunits with phospholipid-G_{M1} bilayers showed that the B subunit associated to and remained on the bilayer surface. In addition, these calorimetric experiments indicated that the toxin insertional mechanism was by a direct penetration of the A subunit into the lipid

bilayer matrix. The insertional event was facilitated under conditions of lateral phase separation induced by calcium. These results are in agreement with toxin thermal denaturation studies using thermal gel analysis and DSC that there is a hydrophobic interaction between the A subunit and the lipophilic matrix of ganglioside G_{M1} micelles and bilayers containing ganglioside G_{M1}.

This study represents the first time that the interactions of a ligand-receptor system have been investigated at this detailed molecular level.

TABLE OF CONTENTS

PART	PAGE
INTRODUCTION	1
Ganglioside-Phospholipid Interactions	5
Cholera Toxin-Ganglioside G_{M1} Interactions	8
REFERENCES	15
I. PURIFICATION OF GANGLIOSIDE G_{M1}	19
Neuraminadase Treatment	20
Ion Exchange Chromatography	21
Silica Gel Chromatography	22
Resorcinol Detection	23
REFERENCES	24
II. LIPID PHASE SEPARATIONS INDUCED BY THE ASSOCIATION OF CHOLERA TOXIN TO PHOSPHOLIPID MEMBRANES CONTAINING GANGLIOSIDE G_{M1}	26
Introduction	27
Materials and Methods	29
Results	35
Discussion	50
REFERENCES	54
III. LATERAL DIFFUSION OF GANGLIOSIDE G_{M1} IN PHOSPHOLIPID BILAYER MEMBRANES	57
Introduction	58
Materials and Methods	59
Results	63

PART PAGE

III. (CONTINUED)

Discussion 75

REFERENCES 79

IV. THERMAL STABILITY AND INTERSUBUNIT INTERACTIONS OF CHOLERA TOXIN IN SOLUTION AND IN ASSOCIATION TO ITS CELL SURFACE RECEPTOR, GANGLIOSIDE G_{M1} 82

Introduction 83

Materials and Methods 85

Results 91

Discussion 109

REFERENCES 115

CONCLUSION 118

VITA 121

LIST OF FIGURES

FIGURE		PAGE
II-1.	Tryptophan emission spectrum of cholera toxin in solution, in the presence of DPPC large unilamellar vesicles containing ganglioside G_{t1b} or after addition of DPPC large unilamellar vesicles containing ganglioside G_{M1}	36
II-2.	Heat capacity function vs. temperature at different Ca^{2+} concentrations for DPPC large unilamellar vesicles containing 10% ganglioside G_{M1}	38
II-3.	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	39
II-4.	Heat capacity profile for DPPC large unilamellar vesicles containing 10 mol % ganglioside G_{M1} and cholera toxin B subunit in the presence of increasing amounts of Ca^{2+}	42
II-5.	Excess heat capacity function versus temperature for DPPC large unilamellar vesicles alone or in the presence of cholera toxin A1 subunit	43
II-6.	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+}	45
II-7.	Phosphorescence emission spectrum of erythrosin isothiocyanate labeled cholera toxin at 20°C . . .	47
II-8.	Time-averaged phosphorescence anisotropy vs. temperture for erythrosin isothiocyanate labeled cholera toxin in the presence of DPPC giant unilamellar vesicles containing 10% ganglioside G_{M1}	49
III-1.	Structure of 5 (((2-Carbohydrazino)methyl)thio)acetyl) amino eosin (CMTAA-eosin) modified ganglioside G_{M1}	65
III-2.	Tryptophan emission spectrum of cholera toxin in the absence or presence of stoichiometric amounts of CMTAA-eosin labeled ganglioside G_{M1}	66

FIGURE PAGE

III-3. Temperature dependence of the lateral mobility parameters for CMTAA-eosin labeled ganglioside G_{M1} incorporated into DMPC bilayers 68

III-4. Lateral diffusion coefficient vs. temperature for DMPC bilayers containing a total of either 1% G_{M1} or 10% G_{M1} in the presence or absence of stoichiometric amounts of cholera toxin 72

III-5. The temperature dependence of excimer formation for pyrene PC incorporated into DPPC vesicles as a function of ganglioside G_{M1} and calcium concentration 74

IV-1. Heat capacity profiles for either cholera toxin or isolated B subunit pentamer free in aqueous solution 92

IV-2. Enlargement of the B subunit transition region from heat capacity profiles of intact cholera toxin incubated with increasing amounts of ganglioside G_{M1} 94

IV-3. Enlargement of the A subunit transition region from calorimetric scans of intact cholera toxin scanned with increasing concentrations of ganglioside G_{M1} 96

IV-4. Dependence of the transition temperature on the concentration of ganglioside G_{M1} for the A and B subunit transitions of cholera toxin 98

IV-5. A series of consecutive DSC scans for isolated B subunit pentamer 100

IV-6. Dependence of the degree of reversibility of the B subunit unfolding transition on the time spent by the sample above the transition temperature in the absence or presence of excess ganglioside G_{M1} . . 101

IV-7. Laser densitometer tracing of differential solubility thermal gel analysis experiment of cholera toxin in solution, in the presence of egg PC small sonicated vesicles, in the presence of ganglioside G_{M1} micelles or in the presence of egg PC small sonicated vesicles containing 10 mol % of ganglioside G_{M1} 103

FIGURE PAGE

IV-8. Normalized peak areas versus temperature calculated from differential solubility gel analysis experiments for cholera toxin alone, in the presence of lipid vesicles, in the presence of ganglioside G_{M1} micelles or in the presence of lipid vesicles containing 10 mol % G_{M1} 105

IV-9. Power output as a function of time for successive injections of intact cholera toxin or isolated B pentamer at equal intervals of 1 hour 108

INTRODUCTION

Any living cell is enclosed by a membrane that confines the interior of the cell and separates it from the external environment (for a review see Harrison & Lunt, 1980). Moreover, eucaryotic cells have additional intracellular membrane-encapsulated organelles which act to compartmentalize various necessary biological functions including metabolic processes, secretion and macromolecular biosynthesis. In each case this boundary protects the internal constituents from variable external influences and allows for the selective passage of certain molecules. In addition, this semipermeable barrier contains various components which enable the cell (organelle) to communicate with its external surroundings and with other cells (organelles).

The structural framework of both the plasma- and intracytoplasmic membranes is that of the lipid bilayer. This bilayer structure is predominately comprised of amphipatic molecules, namely phospholipids, sterols and glycolipids. Not only does the lipid bilayer provide structural integrity, but through the modulation of membrane physical state monitors communication, shape and dynamics of the membrane.

As the other major component of biological membranes, proteins function as catalysts in membrane-associated reactions, transporters of molecules, structural links between cytoskeletal elements and the extracellular matrix, and receptors or receptor-components. Depending on their individual function(s), these proteins either can be situated

on the bilayer surface (peripheral membrane protein) or inserted into the lipid bilayer (integral membrane protein).

The issues surrounding the interplay between lipid molecules with one another and with proteins are complex and are of fundamental importance to understanding the many activities of biological membranes. For example, how are functional biological membranes organized and constantly maintained and what are the molecular mechanisms of membrane biogenesis are major questions still to be answered. These questions raise a more fundamental problem of elucidating the mechanism of insertion of proteins into membranes.

Interactions between lipids, especially phospholipids, have been studied extensively (Silvius, 1982). On the other hand, our understanding of the role of glycolipids, particularly sialic acid containing glycosphingolipids or gangliosides, on membrane structure and function is less understood. Although the chemical structure and cellular distribution of gangliosides have been thoroughly characterized, the biological function and structural organization of gangliosides in membranes is just beginning to be elucidated (Wiegandt, 1985; Curatolo, 1987a, 1987b).

Unlike lipid-lipid interactions, the effects of proteins on lipid bilayer structure and dynamics is not as well characterized. Integral proteins have been found to be sensitive to the nature of the lipid environment to which they are embedded by altering the order and mobility of lipid acyl chains and the lateral organization of the bulk membrane lipid. On the contrary, peripheral proteins, which associate through electrostatic interactions to the lipid headgroup region on

the membrane surface, do not directly perturb the hydrophobic core of the phospholipid bilayer. In addition to the activities with intrinsic and extrinsic membrane proteins, biological membranes must interact with various exogenous substances ranging from whole organisms to small molecules such as ions, drugs, hormones and peptide and protein toxins. As in the case of peripheral proteins, the association of the ligand can be of a general nature or result in the recognition of a specific type of lipid molecule. In the latter case domains can be formed within the bilayer due to the lateral redistribution (lateral phase separation) of the lipid molecules. Those areas in contact with the ligand become enriched with a particular type of lipid while the rest of the membrane becomes devoid of it. Since similar structural elements usually pack better than dissimilar elements, this phenomenon often results in the formation of compositionally separated domains. Finally, despite the important role of membrane lipids for the maintenance of the structural integrity and functionality of membrane-associated proteins, very little is known regarding the effects of lipids, especially gangliosides, on protein structure and function. A major reason for this is the technical problems associated with the purification and membrane reconstitution of integral proteins.

Model membrane systems consisting of a single lipid, two lipid species or pure lipid plus protein are easy to manipulate and have been used to provide insights into the basic interactions between lipids and proteins. Several physicochemical techniques have been

employed in the study of protein-lipid/lipid-lipid interactions using model lipid systems. Differential scanning calorimetry (DSC) is a powerful yet nonperturbing thermodynamic technique for characterizing the thermotropic behavior of lipids and proteins in model systems (Freire et al., 1983; Wiener et al., 1983; Rigell et al., 1986). When combined with differential solubility thermal gel analysis (Rigell & Freire, 1987) a more complete description can be obtained for the individual subunit thermotropic contributions to the thermal denaturation of a soluble or membrane-associated multisubunit protein. Also the use of isothermal reaction calorimetry in thermodynamic studies of biochemical reactions has proved to be useful not only for the direct determination of the thermodynamic parameters (enthalpy and entropy changes), but for the additional analytical information that may be interpreted in terms of the physicochemical properties of the biochemical system under study. Recently this thermodynamic technique has been improved in order to achieve the sensitivity levels necessary to measure protein association and insertion into membranes (Ramsay et al., 1986; Myers et al., 1987). Fluorescence spectroscopy has been used to evaluate both the static and dynamic properties of protein-membrane interactions (Devaux & Seigneuret, 1985). In particular, fluorescence photobleaching recovery (Edidin, 1987) which measures the lipid or protein lateral mobility within the bilayer can be used as a sensitive assay of the various domains in biological and artificial membrane systems.

In this thesis, a biophysical study of the effects of the addition of a glycolipid component to phospholipid bilayers and the

interactions of this model membrane system following the association of two ligands will be addressed. Specifically, the results of a study of the interactions of cholera toxin with its cell surface glycolipid receptor, ganglioside G_{M1} will be presented. First, the effects of this ganglioside-binding protein on membrane physical parameters will be reported. In addition, the effects of calcium on the physical parameters of ganglioside G_{M1}-phospholipid bilayers were studied and used to form asymmetric compositional domains to determine the interaction of cholera toxin to ganglioside G_{M1}-containing membranes following such a perturbation. Finally, studies were performed to further characterize the interactions of ganglioside G_{M1} on cholera toxin structure and function.

1. GANGLIOSIDE-PHOSPHOLIPID INTERACTIONS

Ubiquitous in all vertebrate cells, gangliosides are found in especially high concentrations (~10-15%) in the membranes of the central nervous system (for a general review see Wiegandt, 1985). Gangliosides are embedded in the outer leaflet of the membrane with their sialooligosaccharide portion extended 21 Å from the membrane surface (McDaniel & McIntosh, 1986) toward the extracellular matrix. Because of their cellular distribution and asymmetric membrane localization, it is anticipated that gangliosides play an important role in cellular recognition, antigenicity, and cell growth regulation. Gangliosides have been implicated as surface receptors

for various ligands including the bacterial toxin, cholera toxin (Fishman & Brady, 1976).

Unlike phospholipids which form bilayer systems, gangliosides form micelles in aqueous media at critical micellar concentrations of 10^{-6} M (Masserini & Freire, 1987). General features of the physical properties of gangliosides in water have been reviewed (Wiegandt, 1985; Curatolo, 1987a).

The distribution of gangliosides in a phospholipid environment is under extensive study using mixed model membrane systems. Gangliosides can be codispersed with phospholipids at up to 25 mole percent and still maintain a vesicular structure with both lipid components distributed equally in the two surfaces of the bilayer; at higher concentrations of gangliosides, mixed ganglioside-phospholipid micellar structures are formed (Hill & Lester, 1972). More recently, Felgner et al. (1981) demonstrated that gangliosides can be spontaneously incorporated into the outer leaflet of phospholipid vesicles at up to 13 mole percent ganglioside before vesicular destabilization took place. This second method has the advantage of producing ganglioside-containing phospholipid vesicles which mimic the asymmetric distribution of gangliosides found in the plasma membrane. Calorimetric studies show gangliosides form almost complete ideal mixtures with phosphatidylcholine bilayers (Sillerud et al., 1979). An important factor influencing the molecular organization of gangliosides within the lipid bilayer is the presence of negative charges in the ganglioside polar headgroup. Characterization of the modification of the molecular packing, interfacial conformation, type

of surface phase and electrical potential of phospholipid model systems by the presence of gangliosides has been carried out in several laboratories (McDaniel et al., 1984; Maggio et al., 1978). Direct visualization of ganglioside organization in phospholipid bilayers has been attempted independently by two groups using freeze-etch electron microscopy. The reports produced conflicting results. Peters et al. (1984) described the formation of ganglioside domains within the bilayer while Thompson et al. (1985) observed no clustering of the ganglioside molecules within the lipid bilayers. These latter results are in agreement with our observations using fluorescent gangliosides.

The influence of membrane-associated proteins and exogenous substrates on ganglioside-membrane physical properties such as lipid composition, lipid fluidity and the formation of lipid compositional domains at the membrane surface as of yet is not fully understood. Since the initial DSC studies from this laboratory describing a calcium-induced lateral phase separation of odd-numbered sialic acid containing gangliosides incorporated into phospholipid bilayers, others have studied the modulation of Ca^{2+} on ganglioside-membrane physical state. The overall findings from these studies demonstrated that gangliosides have very little Ca^{2+}-binding affinity (McDaniel & McLaughlin, 1985) and do not redistribute into clusters in phospholipid bilayers due to a preferential chelation of the ganglioside headgroup region by calcium; gangliosides bind calcium as weakly as phospholipids with an association constant of less than 100

M^{-1}. DSC studies (Masserini & Freire, 1986) reported that the Ca^{2+}-induced phase separation was dependent on the chain length difference between the ganglioside long chain base and phosphatidylcholine acyl chains, suggesting that for systems approaching non-ideality of mixing, calcium can induce a ganglioside preference for the fluid phase of the bilayer producing a passive exclusion of ganglioside from the gel-state phospholipid domains as a consequence of a tightening of the Ca^{2+}-perturbed phospholipid bilayer.

In this thesis, the physical parameters for ganglioside G_{M1}-phospholipid bilayers were determined in the absence or presence of two different ligands, calcium and cholera toxin. For these studies DSC was used to monitor the phospholipid acyl chain phase transition by observing a decrease in the enthalpy change and/or lateral phase separation of the different lipid components into distinct domains. The DSC studies showed a calcium-induced lateral redistribution in ganglioside-phospholipid vesicles. To further study this lateral phase separation phenomenon, ganglioside and phospholipid lateral diffusions of the ganglioside-containing model membranes were measured by fluorescence photobleaching recovery or by monitoring the frequency of molecular encounters of the probe molecule, pyrene.

2. CHOLERA TOXIN-GANGLIOSIDE G_{M1} INTERACTIONS

A detailed description of the biochemical and pathological effects of cholera toxin on the cell is outside of the scope of this thesis. For more information concerning these aspects several good

reviews have been published (Gill, 1977; Van Heyningen & Seal, 1983; Van Heyningen, 1985).

Cholera toxin is a globular protein (M = 81,000) secreted by the bacterium Vibrio cholerae (for a review of the protein chemistry, see Lai, 1980). It can be purified readily from culture filtrates of V. cholerae. Today purification occurs on a large industrial scale so the toxin is available from several commercial sources.

Like most bacterial toxins and many plant toxins, cholera toxin is no exception in being composed of two components termed A and B which contribute enzymatic activity and cell surface binding properties, respectively. In the case of cholera toxin, the binding (B) component consists of a noncovalent assembly of five identical subunits (M = 11,000). An isolated aggregate of five B subunits is also secreted by the bacterium and is immunologically indistinguishable from the whole toxin molecule. The A component (M = 26,000) consists of two subunits termed A1 (M = 21,000) and A2 (M = 5,000) which are held via a disulfide linkage. Subunit A1 is an enzyme which is thought to penetrate the plasma membrane of susceptible cells catalyzing the transfer of an ADP-ribose moiety to the N_S regulatory protein component of adenylate cyclase (Dolphin, 1987).

The primary sequence for the entire toxin molecule has been determined (Kurosky et al., 1977; Duffy et al., 1981; Lai, 1977; Duffy et al., 1985). From chemical crosslinking (Gill, 1976) and electron microscopy image reconstruction studies (Ludwig et al., 1986; Ribi & Kornberg, 1987), the toxin molecule is arranged as a symmetrical

pentameric ring of B subunits with the A component lying in the central aqueous channel. Furthermore, since the A2 subunit is necessary for the reconstitution of the holotoxin it is thought to hold the A1 subunit and B subunit pentamer together. This arrangement leaves the A1 subunit exposed on the outside of the molecule as proposed from hydrodynamic studies (Dwyer & Bloomfield, 1982). From these same experiments the dimensions of the toxin molecule were determined.

Cholera toxin interacts with the membrane surface utilizing ganglioside G_{M1} as its receptor. Due to its pentavalent nature, one B pentamer molecule can bind up to five ganglioside G_{M1} molecules. In fact, the oligosaccharide portion of ganglioside G_{M1} is the binding determinant. Removal of either the sialic acid moiety or the terminal galactose portion of oligo-G_{M1} will abolish association. The B component alone exists in nature and has been shown to bind ganglioside G_{M1} with the same specificity and affinity as the holotoxin. A dissociation constant in the order of 10^{-8} - 10^{-9} M has been measured for the binding of oligo-G_{M1} to cholera toxin by Sattler et al. (1978) using equilibrium dialysis. Other authors have determined values close to 10^{-10} M for the binding of ganglioside G_{M1} to intact cells (Gill, 1977).

To date, the binding site of the toxin molecule remains largely undefined at the molecular level. Each B monomer contains a single tryptophan residue. It is known that this tryptophan is in close proximity to the binding site since the fluorescence emission spectrum

is shifted to lower wavelengths in the presence of ganglioside G_{M1} (Mullin et al., 1976) or the oligosaccharide moiety of ganglioside G_{M1} (Fishman et al., 1978), suggesting that following binding the lone tryptophan in the B subunit is in a more hydrophobic environment. From extensive fluorescence experiments, DeWolf et al. (1981) have hypothesized the oligosaccharide binding site to be located at the interface between two adjacent B subunit monomers and that the tryptophan is located in this region. Ludwig et al. (1985) used monoclonal antibodies to map amino acids including tryptophan which may mediate oligosaccharide/ganglioside binding.

The toxin-ganglioside association is believed to cause a conformational change in the protein that facilitates the exposure and subsequent penetration of the A subunit into the membrane. In addition to the intrinsic protein fluorescence studies, techniques ranging from DSC (Dalziel et al., 1984), circular dichroism (Fishman et al., 1978), and NMR (Sillerud et al., 1981) have been utilized to study this conformational change.

Although it is acknowledged that the A subunit or at least part of the enzymatically active A1 portion must traverse the membrane to gain access to adenylate cyclase bound to the cytoplasmic side of the membrane, the precise mechanism of membrane penetration is not known. As of today, the most widely accepted theory of how the A subunit is transported across the membrane is that membrane penetration occurs by a direct interaction of the A subunit with the hydrophobic interior of the bilayer. Wisnieski and Bramhall (1981) provided the first indirect evidence that the toxin by itself could direct the

penetration of the A subunit into the lipid bilayer. Using a photoactivable glycolipid probe incorporated into viral membranes, they noted entry of only the A subunit within seconds of toxin binding. The topology of the interaction of cholera toxin with ganglioside G_{M1} micelles (Tomasi & Montecucco, 1981) and ganglioside G_{M1}-containing liposomes (Tomasi et al., 1982) has also been monitored by the hydrophobic photolabelling technique. In the case of ganglioside micelles both subunits A1 and A2 were labeled, but not the B subunit. When the probe was incorporated into G_{M1}-containing liposomes, Tomasi et al. (1982) showed that only the A1 portion was labeled and that the labeling occurred only after the reduction of the A1-A2 disulfide bridge. In addition, results from quasi-elastic light scattering experiments have been interpreted in terms of a model in which the B subunits are absorbed and remain on the lipid bilayer surface while the A1 fragment is incorporated into the hydrophobic interior (Dwyer & Bloomfield, 1982). From these studies, the role of the reduction of the A subunit on membrane penetration remains uncertain with only the study of Tomasi et al. (1982) concluding reduction as a requirement for penetration. More recently, three-dimensional structural studies at 15 Å resolution (Ribi & Kornberg, 1987) have revealed that the A subunit initially does insert into the hydrophobic core of the lipid bilayer and that upon reduction of the A subunit there is a further penetration of the enzymatically active A1 subunit.

In this thesis, the interaction of cholera toxin with

ganglioside G_{M1} has been studied to determine the effects on membrane physical state of a soluble protein following membrane association to a specific ganglioside receptor, to characterize the subsequent insertion of the protein into receptor-containing membranes and to define the role of the ganglioside molecule in this interaction by measuring the effect of ganglioside association on the structure and function of the cholera toxin molecule. In these studies, we used DSC to monitor the thermotropic behavior of G_{M1}-containing phospholipid bilayers following the addition of cholera toxin or its isolated subunits. These studies showed that cholera toxin as a multisubunit protein exerts distinct effects on the thermotropic behavior of the G_{M1}-phospholipid bilayers and that these effects could be correlated to the individual subunits. Additional evidence of the effect of the association and insertion of cholera toxin on membrane physical state was obtained from steady-state and time-resolved fluorescence techniques. Tryptophan emission spectroscopy was used to determine the effects of lipid fluidity of G_{M1}-phospholipid bilayers on toxin association. Also the dynamics of the toxin-membrane interaction was monitored by measuring the rotational mobility of toxin bound to G_{M1}-phospholipid bilayers using phosphorescence spectroscopy. The effect of toxin association on lipid lateral mobility and ganglioside lateral mobility was measured using the pyrene excimer technique and fluorescence photobleaching recovery, respectively. In addition to the studies measuring the effects of cholera toxin on membrane physical state, a study of the effect of ganglioside association on cholera toxin structure was carried out. We used DSC to study this

interaction by monitoring the Tm, enthalpy change and cooperative unfolding of the protein structural transition in the absence and presence of ganglioside G_{M1}. The DSC results revealed that the addition of ganglioside had a stabilizing effect on the thermotropic behavior of the cholera toxin molecule. Additional evidence of the thermal stabilization by ganglioside G_{M1} was obtained using differential solubility thermal gel analysis. Finally, direct measurement of the energetics of the sequence of events comprising the association and insertion of cholera toxin into the membrane was determined using isothermal reaction calorimetry.

INTRODUCTION

REFERENCES

REFERENCES

Curatolo, W. (1987a) Biochim. Biophys. Acta 906: 111-136.

Curatolo, W. (1987b) Biochim. Biophys. Acta 906: 137-160.

Dalziel, A. W., Lipka, G., Chowdhry, B. Z., Sturtevant, J. W., and Schafer, D. E. (1984) Mol. Cell. Biochem. 63: 83-91.

Devaux, P. E., and Seigneuret, M. (1985) Biochim. Biophys. Acta 822: 63-125.

DeWolf, M. J. S., Fridkin, M., Epstein, M., and Kohn, L. D. (1981) J. Biol. Chem. 256: 5481-5488.

Dolphin, A. C. (1987) Trends Neurosci 10: 53-57.

Duffy, L. K., Kurosky, A., and Lai, C. Y. (1985) Arch. Biochem. Biophys. 239: 549-555.

Duffy, L. K., Peterson, J. W., and Kurosky, A. (1981) J. Biol. Chem. 256: 12252-12256.

Dwyer, J. D., and Bloomfield, V. A. (1982) Biochemistry 21: 3231-3234.

Edidin, M. (1987) in Current Topics in Membranes and Transport (Klausner, R. D., Kempf, C., and van Renswoude, J., Eds.) vol. 29, pp. 91-127. Academic Press, Inc., Orlando.

Felgner, P. L., Freire, E., Barenholz, Y., and Thompson, T. E. (1981) Biochemistry 20: 2168-2172.

Fishman, P. H., and Brady, R. O. (1976) Science 197: 906-915.

Fishman, P. H., Moss, J., and Osborne, J. C., Jr. (1978) Biochemistry 17: 711-716.

Freire, E., Markello, T., Rigell, C., and Holloway, P. W. (1983) Biochemistry 22: 1675-1680.

Gill, D. M. (1976) Biochemistry 15: 1242-1248.

Gill, D. M. (1977) Adv. Cyclic Nucleotide Res. 8: 85-118.

Harrison, R., and Lunt, G. G. (1980) Biological Membranes: Their Structure and Function. Blackie & Sons Limited, Glasglow.

Hill, M. W., and Lester, R. (1972) Biochim. Biophys. Acta 282: 18-30.

Kurosky, A., Markel, D. E., and Peterson, J. W. (1977) J. Biol. Chem. 252: 7257-7264.

Lai, C. Y. (1977) J. Biol. Chem. 252: 7249-7256.

Lai, C. Y. (1980) CRC Crit. Rev. Biochem. 8: 171-206.

Ludwig, D. S., Holmes, R. K., and Schoolnik, G. K. (1985) J. Biol. Chem. 260: 12528-12534.

Ludwig, D. S., Ribi, H. O., Schoolnik, G. K., and Kornberg, R. D. (1986) Proc. Natl. Acad. Sci. U.S.A. 83: 8585-8588.

Maggio, B., Cumar, F. A., and Caputto, R. (1978) Biochem. J. 175: 1113-1118.

Masserini, M., and Freire, E. (1986) Biochemistry 25: 1043-1049.

Masserini, M., and Freire, E. (1987) Biochemistry 26: 237-242.

McDaniel, R. V., and McIntosh, T. J. (1986) Biophys. J. 49: 94-96.

McDaniel, R. V., McLaughlin, A., Winiski, A. P., Eisenberg, M., and McLaughlin, S. (1984) Biochemistry 23: 4618-4624.

McDaniel, R. V., and McLaughlin, S. (1985) Biochim. Biophys. Acta 819: 153-160.

Mullin, B. R., Aloj, S. M., Fishman, P. H., Lee, G., Kohn, L. D., and Brady, R. O. (1976) Proc. Natl. Acad. Sci. U.S.A. 73: 1679-1683.

Myers, M., Mayorga, O. L., Emtage, J., and Freire, E. (1987) Biochemistry 26: 4309-4315.

Peters, M. W., Mehlhorn, I. E., Barber, K. R., and Grant, C. W. M. (1984) Biochim. Biophys. Acta. 778: 419-428.

Rahmann, H., Probst, W., and Muhleisen, M. (1982) Japan J. Exp. Med. 52: 275-286.

Ramsay, G., Prabhu, R., and Freire, E. (1986) Biochemistry 25: 2265-2270.

Ribi, H. O., and Kornberg, R. D. (1987) Biophys. J. 51: 168a.

Rigell, C., de Saussure, C., and Freire, E. (1985) Biochemistry 24: 5638-5646.

Rigell, C., and Freire, E. (1987) Biochemistry 26: 4366-4371.

Sattler, J., Schwarzmann, G., Kanck, I., Rohm, K. H., and Wiegandt, H. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359: 719-723.

Sillerud, L. O., Prestegard, J. H., Yu, R. K., Konigsberg, W. H., and Schafer, D. E. (1981) J. Biol. Chem. 256: 1094-1097.

Sillerud, L. O., Schafer, D. E., Yu, R. K., and Konigsberg, W. H. (1979) J. Biol. Chem. 21: 10876-10880.

Silvius, J. R. (1982) in Lipid-Protein Interactions (Jost, P. and Griffith, O. H., Eds.) vol. II, pp. 239-276, John Wiley & Sons, Inc., New York.

Thompson, T. E., Johnson, M., Alietta, M., Brown, R. E., and Tillack, T. W. (1985) Biochim. Biophys. Acta 817: 229-237.

Tomasi, M., and Montecucco, C. (1981) J. Biol. Chem. 256: 11177-11181.

Tomasi, M., D'Agnolo, G., and Montecucco, C. (1982) Biochim. Biophys. Acta 692: 339-344.

Van Heyningen, S. (1983) in Current Topics in Membranes and Transport (Kleinzieler, A., and Martin, B. R., Eds.) vol. 18, pp. 445-471, Academic Press, New York.

Van Heyningen, W. E., and Seal, J. R. (1983) Cholera: The American Scientific Experience 1947-1980. Westview Press, Boulder, CO.

Wiegandt, H. (1985) in New Comprehensive Biochemistry (Neuberger, A., and van Deenen, L. L. M., Eds.) vol. 10, pp. 199-260, Elsevier, Amsterdam.

Wiener, J., Wagner, R. R., and Freire, E. (1983) Biochemistry 22: 6117-6123.

Wisnieski, B., and Bramhall, J. S. (1981) Nature (London) 289: 319-321.

PART I

PURIFICATION OF GANGLIOSIDE G_{M1}

PURIFICATION OF GANGLIOSIDE G_{M1}

Ganglioside G_{M1} was purified from di- and trisialoganglioside mixtures which were originally isolated from the Folch extract of bovine brain (Avanti Biochemicals, Birmingham, AL) as outlined in Myers et al. (1984). The purification protocol developed is based on that described by Thompson et al. (1985). Purification of ganglioside G_{M1} includes three major steps: 1) cleavage of sialic acid residues from polysialogangliosides using Neuraminadase, 2) ion exchange chromatography to separate the newly-formed single-charged gangliosides from residual gangliosides and 3) further separation of ganglioside G_{M1} from a crude monosialoganglioside fraction using silica gel chromatography.

1. NEURAMINADASE TREATMENT

Mixed di- and trisialogangliosides (0.5 g) and 1g of egg phosphatidylcholine (Avanti Biochemicals, Birmingham, AL) were dissolved in $CHCl_3$:CH_3OH (1:2), N_2 dried and reconstituted with 80 ml of 20 mM sodium acetate buffer, pH 5.6 containing 0.02% NaN_3. A total of ten units of Neuraminadase (Clostridium perfringens type VI, Sigma, St. Louis, MO) in two aliquots was added to this ganglioside-lipid mixture and then incubated at 37°C for 24 hours. For optimal activity, the Neuraminadase should be reconstituted at a protein concentration of not less than 1.6 mg/ml in 10 mM sodium phosphate buffer pH 6.0 containing 45 mM KCl and 0.3 mg/ml bovine serum albumin (Cassidy et al., 1966). Following enzymatic hydrolysis the sample was

lyophilized, redissolved in a small volume of water, dialyzed (Spectrapor dialysis tubing #3) extensively to remove all salts, and again lyophilized.

2. ION EXCHANGE CHROMATOGRAPHY

DEAE-Sephadex A-25-120 (acetate form) was used in this purification step. To generate the acetate form of DEAE-Sephadex, a given amount of Sephadex was swollen in a 6.5-fold excess (w/v) of 0.5 M ammonium acetate in methanol. After gently shaking the gel slurry for 48 hours to ensure acetate exchange, the excess ammonium acetate was removed by washing the gel in a sintered glass funnel with approximately 100 ml of each of the following series of solvents: CH_3OH, CH_3OH:H_2O (80:20), CH_3OH:H_2O (50:50), H_2O, CH_3OH:H_2O (50:50), CH_3OH:H_2O (80:20) and finally CH_3OH. The gel was stored in excess methanol at 4^oC until needed.

The lyophilized ganglioside-lipid sample was dissolved in a minimal amount of $CHCl_3$:CH_3OH (1:1) and added to the DEAE-Sephadex. Additional chloroform was then added such that the final $CHCl_3$:CH_3OH solvent ratio was 1:1. This gel slurry was allowed to shake at least 2 hours at room temperature but normally was left overnight. Following ganglioside adsorption, the slurry was brought to a final solvent concentration of $CHCl_3$:CH_3OH:H_2O (30:60:8).

The DEAE-ganglioside slurry was packed in a 0.9 x 24 cm column, washed with 3 column volumes of $CHCl_3$:CH_3OH:H_2O (30:60:8) followed by one column volume methanol and finally eluted with 500 ml of 75 mM ammonium acetate in methanol (20 ml fractions). Monosialogangliosides

are preferentially eluted at this salt concentration. The remaining adsorbed gangliosides were washed from the column with 500 ml of 500 mM ammonium acetate in methanol and collected in 100 ml fractions. In both cases the fractions were checked for ganglioside using resorcinol reagent. Resorcinol-positive fractions were combined, evaporated using a flash evaporator, redissolved in $CHCl_3$:CH_3OH (1:1) and spotted on an E. Merck silica gel 60 plate (Alltech Associates, Deerfield, IL). The plate was developed in $CHCl_3$:CH_3OH:0.02% $CaCl_2$ (55:45:10) (Kundu, 1981). An alternate TLC solvent system was n-propanol:0.2% $CaCl_2$ (8:2). Gangliosides were detected with resorcinol and compared to ganglioside standards obtained from Supelco (Bellefonte, PA).

3. SILICA GEL CHROMATOGRAPHY

The Anasil S silica gel (Analabs, New Haven, CT) was prepared for this separation step by dissolving a given amount of gel (120 g for 500 mg of ganglioside) in methanol, removing the fines and air drying the gel to remove the residual methanol. The gel was then activated prior to use by heating to 100°C for 1 hour. Next 300 ml of $CHCl_3$:CH_3OH:H_2O (65:25:4) was added to the cooled gel to form a thick slurry. The gel slurry was poured into a 2.5 x 40 cm column and equilibrated with 2 column volumes of $CHCl_3$:CH_3OH:H_2O (65:25:4).

The next day all monosialoganglioside containing fractions from the ion exchange step were dissolved in $CHCl_3$:CH_3OH:H_2O (65:25:4) and loaded onto the equilibrated silica gel column. The gangliosides

were eluted from the column with 400 ml of the following series of $CHCl_3$:CH_3OH:H_2O (65:25:4), (60:30:5), (60:35:8), (60:40:10) and finally pure methanol. For each solvent ratio except pure methanol, 20 ml fractions were collected; the eluate was collected and discarded in the case of methanol. Every fifth column fraction was evaporated and redissolved in 2 ml of distilled water. An aliquot of these fractions were checked for specific ganglioside species using thin layer chromatography as described above. The fractions containing ganglioside G_{M1} were evaporated, redissolved in water, pooled and dialyzed extensively against water at 4°C. Following dialysis the sample was lyophilized and stored in powder form at -20°C. The yield from 500 mg of crude gangliosides was between 100-150 mg pure G_{M1}.

4. RESORCINOL DETECTION

A qualitative estimation (Svennerholm, 1957) of sialic acid in the column fractions was performed using resorcinol reagent (2 ml of 2% resorcinol, 16 ml of concentrated HCl, 0.05 ml of 0.1 M $CuSO_4 \cdot 5H_2O$ and 1.95 ml of H_2O). A 0.4 ml aliquot of the sample was mixed with 0.4 ml of the reagent and heated to 100°C for 15 minutes. The samples were then visually inspected for the appearance of a blue-violet color.

To detect sialic acid containing gangliosides on developed TLC plates, the plates were allowed to air dry and then sprayed lightly with resorcinol reagent. The plates were covered with a glass plate and heated to 120°C for 15 minutes or until the appearance of blue-violet bands was noted.

PART I

REFERENCES

REFERENCES

Cassidy, J. T., Jourdian, G. M. and Roseman, S. (1966) Methods Enzymol. 8: 680-685.

Kundu, S. K. (1981) Methods Enzymol. 72: 185-204.

Myers, M., Wortman, C., and Freire, E. (1984) Biochemistry 23: 1442-1448.

Svennerholm, L. (1957) Biochim. Biophys. Acta 24: 604-611.

Thompson, T. E., Johnson, M., Allietta, M., Brown, R. E., and Tillack, T. W. (1985) Biochim. Biophys. Acta 817: 229-237.

PART II

LIPID PHASE SEPARATIONS INDUCED BY THE ASSOCIATION OF CHOLERA TOXIN TO PHOSPHOLIPID MEMBRANES CONTAINING GANGLIOSIDE G_{M1}

LIPID PHASE SEPARATIONS INDUCED BY THE ASSOCIATION OF CHOLERA TOXIN TO PHOSPHOLIPID MEMBRANES CONTAINING GANGLIOSIDE G_{M1}

1. INTRODUCTION

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 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. Part II of this thesis addresses this question. In this section, 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 11000 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 21000 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 cell membrane using ganglioside G_{M1} as a receptor.

The studies presented in this section 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.

2. 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 15000g 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 15000g 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 et al., 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 A/D 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 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 ($G = I_{\parallel}/I_{\perp}$).

3. 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. These results are shown in Figure II-1. 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

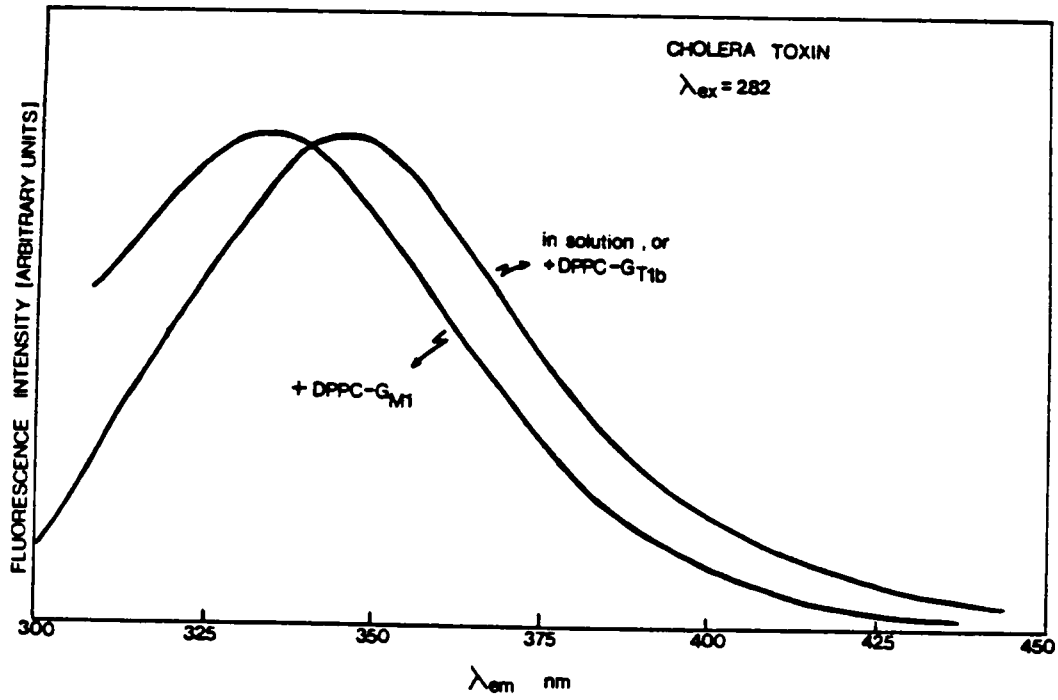

Figure II-1. Tryptophan emission spectrum of cholera toxin in solution, in the presence of DPPC large unilamellar vesicles containing ganglioside G_{T1b} or after addition of DPPC large unilamellar vesicles containing ganglioside G_{M1}.

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 II-2. 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), reported a qualitatively similar pattern for the trisialo-ganglioside G_{t1b}.

The calorimetric results for DPPC-G_{M1} vesicles in the presence of intact cholera toxin are shown in Figure II-3. 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

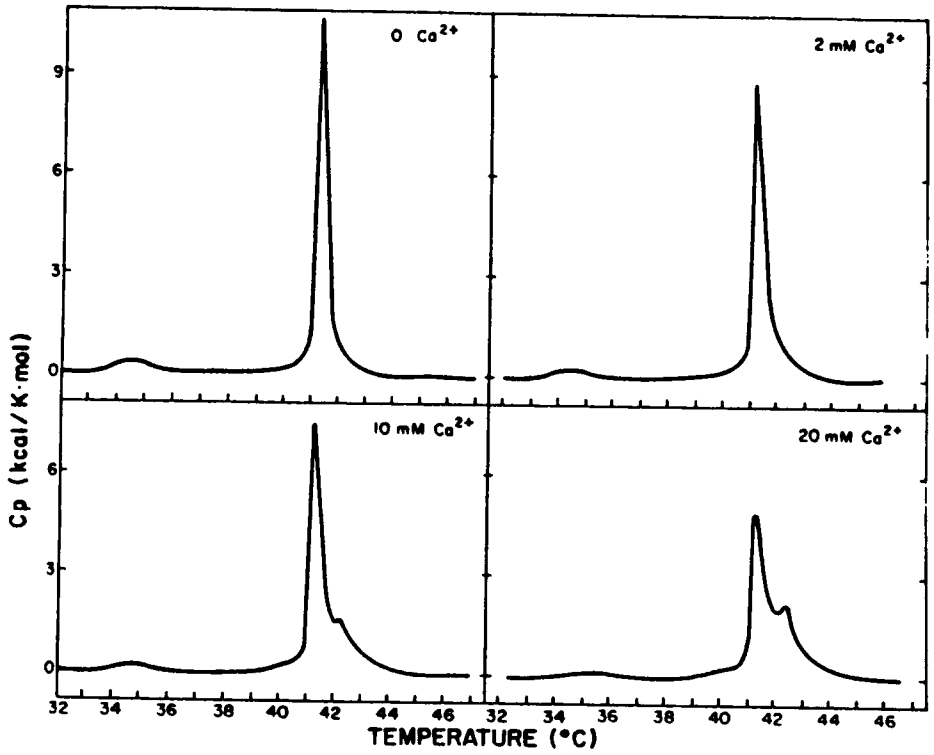

Figure II-2. Heat capacity function vs. temperature at different Ca^{2+} concentrations for DPPC large unilamellar vesicles containing 10% ganglioside G_{M1}.

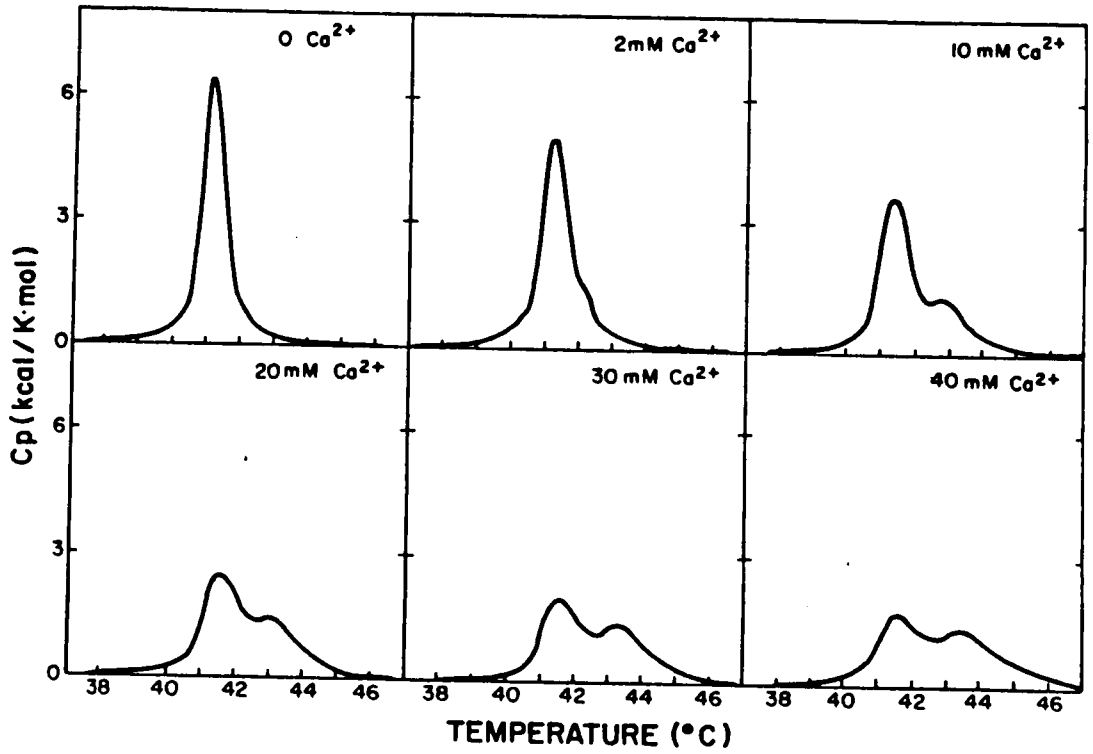

Figure II-3. Heat capacity function vs. temperature at different Ca^{2+} concentrations for DPPC large unilamellar vesicles containing 10% ganglioside G_{M1} in the presence of cholera toxin. The protein to lipid ratio was 1/210.

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. As shown in Figure II-4, 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+}. As depicted in Figure II-5, 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

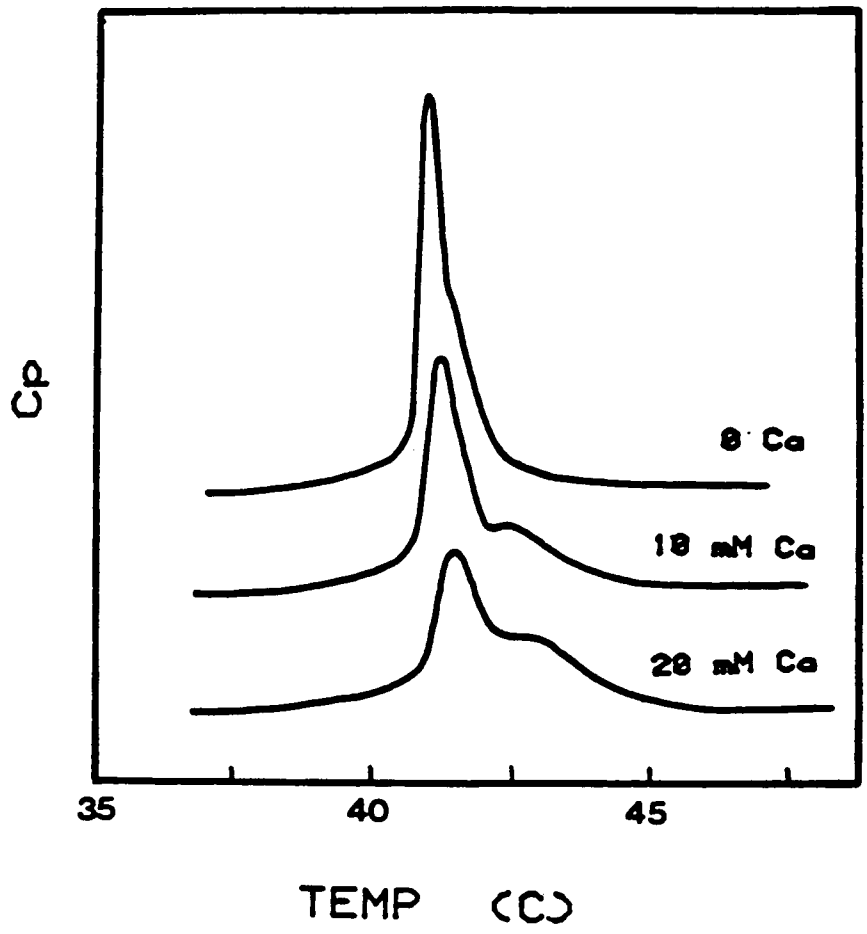

Figure II-4. Heat capacity profile for DPPC large unilamellar vesicles containing 10 mol % ganglioside G_{M1} and cholera toxin B subunit in the presence of increasing amounts of Ca^{2+}.

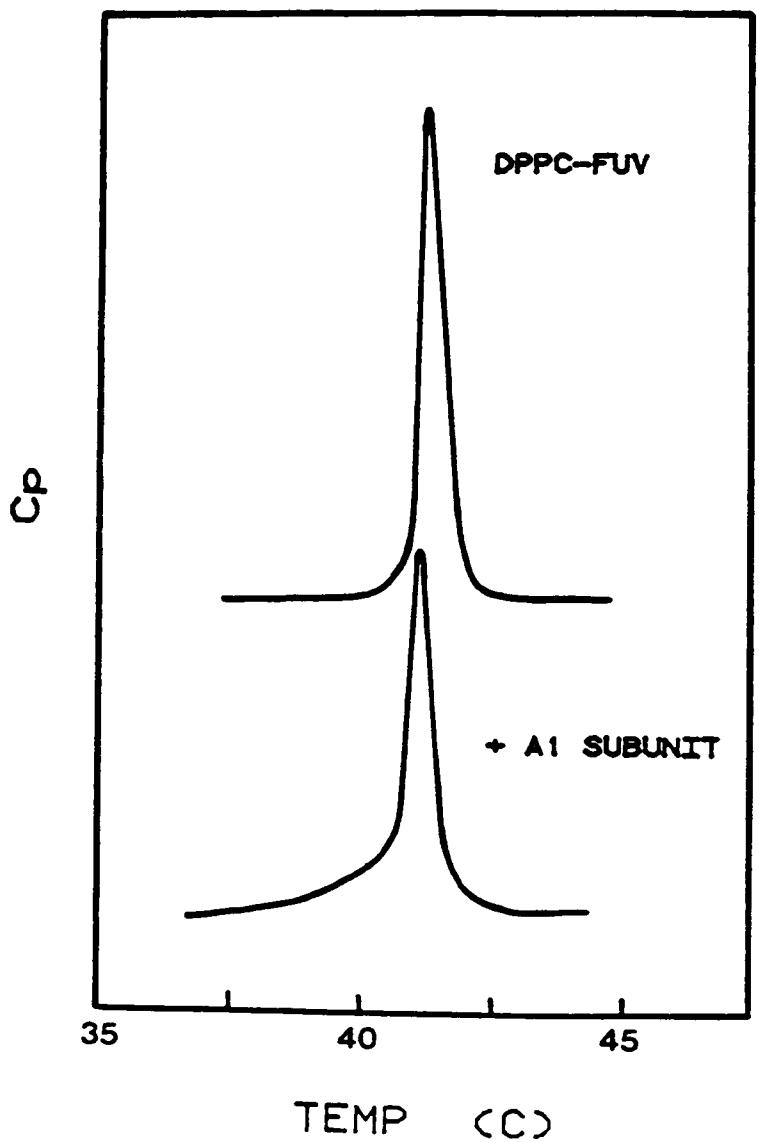

Figure II-5. Excess heat capacity function versus temperature for DPPC large unilamellar vesicles alone or in the presence of cholera toxin A1 subunit.

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, i.e., above the lipid phase transition temperature. As shown in Figure II-6, 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 II-6. 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

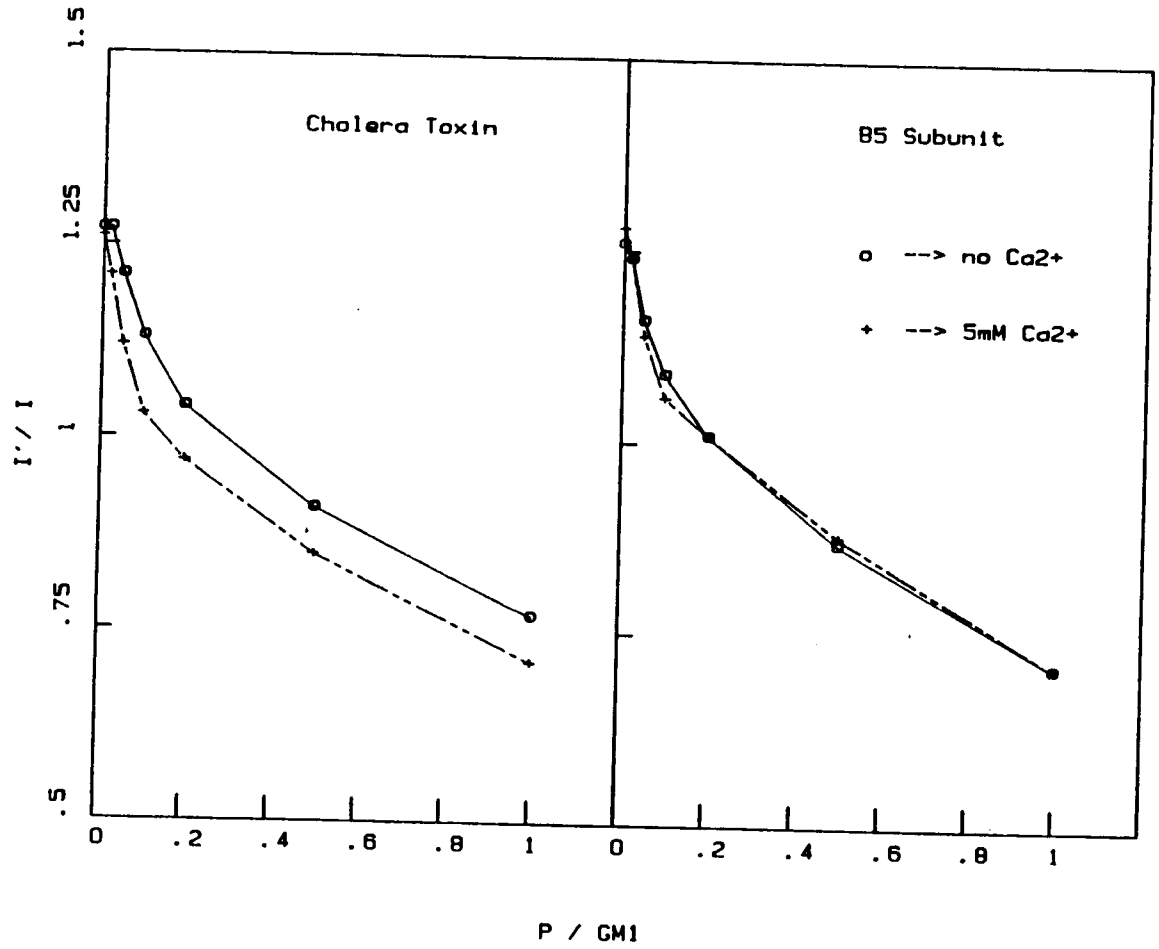

Figure II-6. Excimer/monomer ratios of pyrenephosphatidylcholine incorporated into large unilamellar DPPC vesicles containing 10% G_{M1} vs. increasing concentrations of intact cholera toxin or B subunit in the presence or absence of 5 mM C^{2+}. The samples were excited at 320 nm, and the emissions 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.

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; Babbitt 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 II-7 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 Figure II-7, 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 II-7) of erythrosin-labeled cholera toxin is 250 μs, i.e., somewhat shorter than the lifetime of

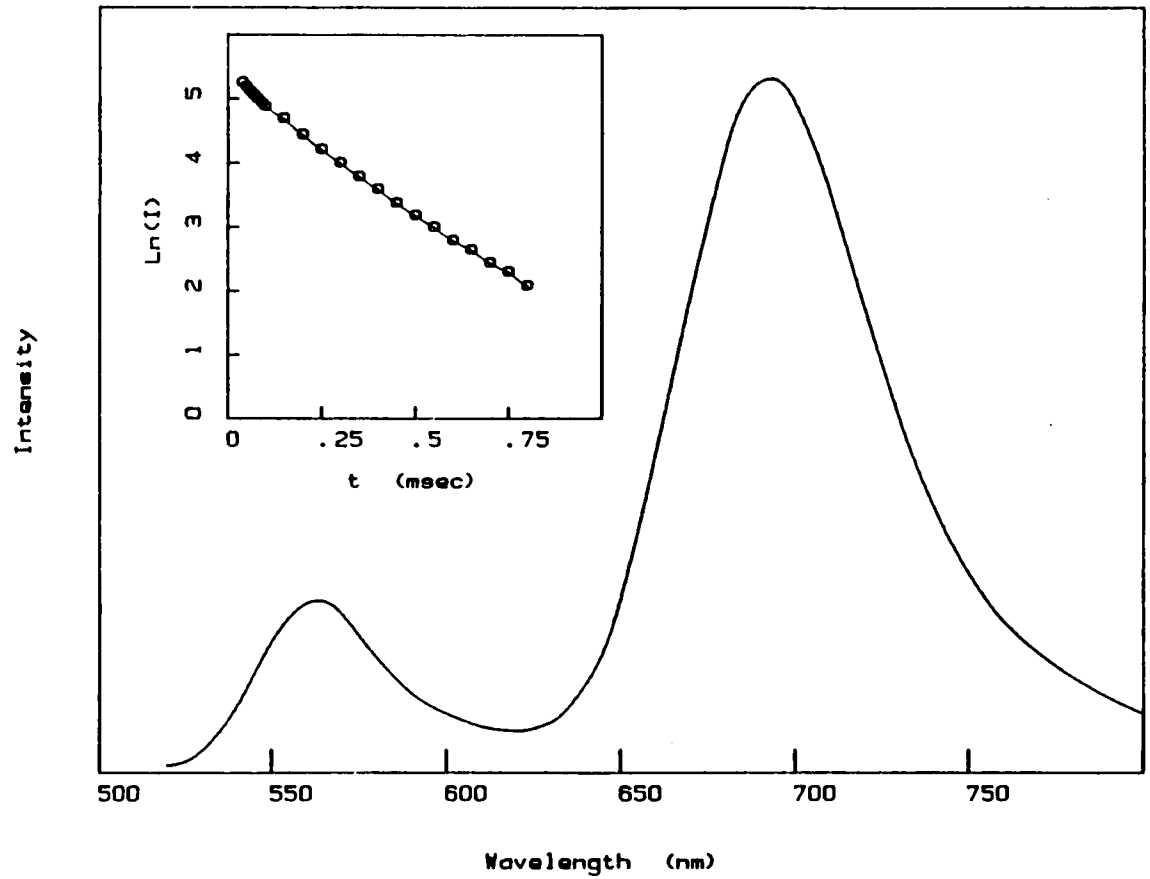

Figure II-7. Phosphorescence emission spectrum of erythrosin isothiocyanate labeled cholera toxin at 20°C. The excitation wavelength was 510 nm. Inset: Natural logarithm of the phosphorescence intensity (690 nm) vs decay time for the labeled cholera toxin. The lifetime of the probe was 0.25 ms.

320 μs obtained by us (Babbitt 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 83000-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 II-8 shows the results 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-linking following incubation

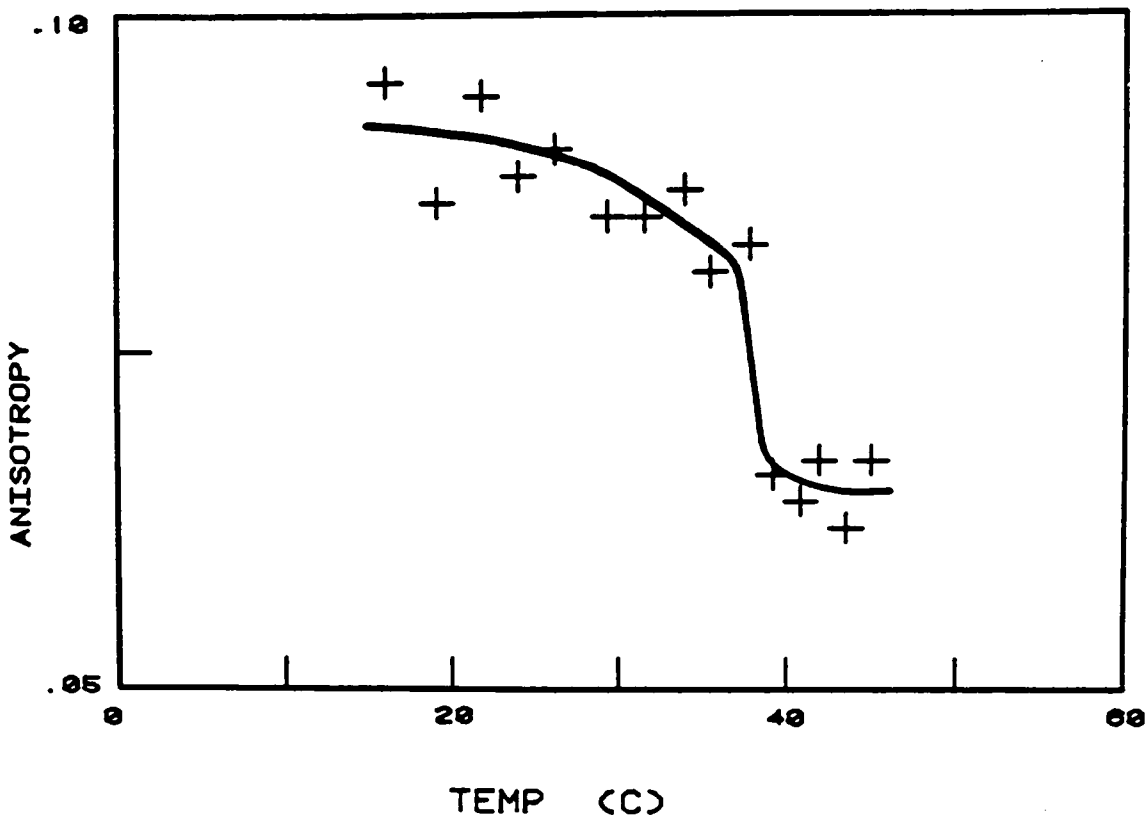

Figure II-8. Time-averaged phosphorescence anisotropy vs. temperature for erythrosin isothiocyanate labeled cholera toxin in the presence of DPPC giant unilamellar vesicles containing 10% ganglioside G_{M1}. The phosphorescence anisotropy was integrated from T_0 = 0.05 ms to T_f = 8 ms after the light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

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.

4. 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 21000-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 II-2, page 39, 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 [see Part III of this thesis for more details) 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.

PART II

REFERENCES

REFERENCES

Babbitt, B., Huang, L., and Freire, E. (1984) Biochemistry 23: 3920-3926.

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

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

Curatolo, W., Sakura, J. D., Small, D. N., and Shipley, G. G. (1977) Biochemistry 16: 2313-2319.

Dwyer, J. D., and Bloomfield, V. A. (1982) Biochemistry 21: 3227-3231.

Felgner, P. L., Freire, E., Barenholz, Y., and Thompson, T. E. (1981) Biochemistry 20: 2168-2172.

Fishman, P. H. (1982) J. Membr. Biol. 69: 85-97.

Fishman, P. H., Moss, J., and Osborne, J. C. (1978) Biochemistry 17: 711-716.

Freire, E., Markello, T., Rigell, C., and Holloway, P. W. (1983) Biochemistry 22: 1675-1680.

Galla, H. J., and 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., and 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., and Esmann, M. (1982) Biophys. J. 37: 265-271.

Moore, C., Boxer, D., and Garland, P. (1979) FEBS Lett. 108: 161-166.

Moss, J., and Vaughn, M. (1979) Annu. Rev. Biochem. 48: 560-581.

Murray, E. K., Restall, C. J., and Chapman, D. (1983) Biochim. Biophys. Acta 732: 347-351.

Myers, M., Wortman, C., and Freire, E. (1984) Biochemistry 23: 1442-1448.

Oku, N., and MacDonald, R. C. (1983) Biochemistry 22: 855-863.

Pownall, J. H., Pao, Q., Hickson, D., Sparrow, J. T., Kusserow, S. K., and Massey, J. B. (1981) Biochemistry 20: 6630-6635.

Schullery, S. E., Schmidt, C. F., Felgner, P., Tillack, T. W., and Thompson, T. E. (1980) Biochemistry 19: 3919-3923.

Sillerud, L. O., Schafer, D. E., Yu, R. K., and 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., and Montecucco, E. (1981) J. Biol. Chem. 256: 11177-11181.

Tuszynski, G. P., Knight, L., Piperno, J. R., and Walsh, P. N. (1980) Anal. Biochem. 106: 118-122.

Wiener, J., Wagner, R. R., and Freire, E. (1983) Biochemistry 22: 6117-6123.

Wong, M., Anthony, F. H., Tillack, T. W., and Thompson, T. E. (1982) Biochemistry 21: 4126-4132.

PART III

LATERAL DIFFUSION OF GANGLIOSIDE G_{M1} IN PHOSPHOLIPID BILAYER MEMBRANES

LATERAL DIFFUSION OF GANGLIOSIDE G_{M1} IN PHOSPHOLIPID BILAYER MEMBRANES

1. INTRODUCTION

Gangliosides are sialic acid-containing glycosphingolipids which are associated exclusively with the outer leaflet of the plasma membrane and synaptic membrane (Steck & Dawson, 1974; Hansson et al., 1977; Ledeen, 1978). Because their oligosaccharide portion is exposed on the cell surface, this class of glycolipids is thought to be important in cell growth regulation and cellular interactions (Hakomori, 1981). Gangliosides have been implicated as cellular recognition sites (Fishman & Brady, 1976) for viruses, protein hormones, and bacterial toxins, of which cholera toxin is the best documented example (Fishman, 1982). Studies with cholera toxin-ganglioside G_{M1} have shown that internalization of the toxin molecule is by receptor-mediated endocytosis and that lateral movement of the complex is an intermediate step (Jeng, 1983). Reports of lateral redistribution induced by cholera toxin in lymphocytes by both indirect immunofluorescence with labeled toxin antibody (Revesz & Greaves, 1975) and by incorporation of exogenous fluorescently labeled gangliosides have been previously reported in the literature (Sedlacek et al., 1976; Spiegel et al., 1984a, 1984b). A major cellular interaction with which gangliosides have been linked is that of synaptic transmission (Tettamanti et al., 1979). The divalent cation Ca^{2+} is thought to have a part in this function by complexing with the negatively charged head group of the ganglioside thereby destabilizing

the synaptic membrane to release its transmitters (Rahmann et al., 1982). The association between sialic acid and Ca^{2+} has been well documented (Jaques et al., 1977; Behr & Lehn, 1973). Previous calorimetric studies have revealed that millimolar amounts of Ca^{2+} can induce solid phase separation in dipalmitoylphosphatidylcholine-fused unilamellar vesicles containing ganglioside G_{M1} (Goins & Freire, 1985) or ganglioside Gt_{1b} (Myers et al., 1984).

In Part III of this thesis, we have studied the role that the head group region of ganglioside G_{M1} plays in its lateral diffusion within the bilayer. Using fluorescence photobleaching recovery we have quantitatively determined the lateral mobility of fluorescently labeled ganglioside G_{M1} incorporated into preformed dimyristoyl phosphatidylcholine vesicles. This method of incorporation allows for an assymmetrical distribution of the gangliosides which mimics that found in the plasma membrane (Felgner et al., 1981). The fluorescence photobleaching recovery experiments have been complemented by excimer formation experiments using pyrene phosphatidylcholine as a probe to measure molecular collisions within the lipid bilayer matrix.

2. MATERIALS AND METHODS

Materials

Ganglioside G_{M1} prepared by sialidase treatment of the crude ganglioside fraction from beef brain was a kind gift of Dr. T. E. Thompson (University of Virginia). The fatty acid composition of this ganglioside G_{M1} is primarily C 18:0 (> 80%), the rest being primarily

a mixture of C 20:0, C 22:0 and C 24:0 fatty acid chains. The aldehyde reactive probe 5(((2-Carbohydrazino) methyl) thio) acetyl) amino eosin (CMTAA-eosin) was a generous gift from Richard Haugland (Molecular Probes, Junction City, OR). Dihexyloxacarbocyanine iodide (DiI) was purchased from Boehringer Mannaheim (Indianapolis, IN). Cholera toxin was purchased from Sigma Chemical Company (St. Louis, MO). The protein was supplied as a lyophilized powder and was reconstituted with distilled water at a concentration of 0.5 mg/ml in 50mM Tris-HCl, 0.2M NaCl, 3mM NaN_3, 1 mM Na_2EDTA, pH 7.5. Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) were obtained from Avanti Biochemicals (Birmingham, AL). 1-Palmitoyl-2-pyrenedecanoylphosphatidylcholine (pyrene-PC) was obtained from KSV Chemicals, (Helsinki, Finland). The purity of all lipids was checked by thin layer chromatography.

Ganglioside Labeling with CMTAA-Eosin

The method of Cherry et al. (1980) was used to label the sialic acid moiety of ganglioside G_{M1}. The ganglioside was first dissolved in 2 ml PBS buffer, pH 7.4. An additional 2 ml PBS buffer containing 2mM sodium periodate was added to the ganglioside solution to give a final concentration of 1 mg/ml. After a 30 minute incubation on ice, the periodate was removed by an overnight dialysis using Spectrapor dialysis tubing (MW cutoff 3500) against PBS buffer pH 7.4 at $4^{o}C$. The following day a 1.5 M excess of CMTAA-eosin was added and the solution incubated for 2 hours on ice in the dark. To remove the excess label, the conjugated ganglioside was dialyzed against PBS

buffer at 4°C in the dark for 2 days with a change of buffer. Following dialysis, the sample was passed through a G-25 column using a rapid filtration-centrifugation procedure (Tuszynski, et al., 1980). To check for the presence of the labeled ganglioside a TLC plate (Kundu, 1981) was run. An aliquot of the sample was N_2 dried and redissolved in chloroform:methanol (1:1) and spotted on an E. Merck silica gel 60 plate (Alltech Associates, Deerfield, IL). The plate was developed in chloroform:methanol:0.02% $CaCl_2$ (55:45:10). After air drying, the pink bands which migrated with standard G_{M1} were detected by either spraying the plate with primulin or resorcinol (Kundu, 1981). To separate the labeled ganglioside from any additional unconjugated probe preparative TLC plates were run under the same conditions described above. The labeled gangliosides were eluted from the silica gel by adding excess chloroform:methanol: distilled H_2O (5:5:1), vortexed vigorously, and allowed to sit for at least 30 minutes (Kundu, 1981). The solution was centrifuged 10 minutes at 3000 rpm and the labeled gangioside containing supernatant was removed. The solvent was evaporated under N_2. To remove any excess silica gel, 0.5 ml of chloroform:methanol (1:1) was added to the dried sample and recentrifuged. The solvent was again removed and the labeled ganglioside redissolved in PBS buffer and stored at 4°C. The concentration of label could be measured by using the extinction coefficient of eosin at 540 nm of 83 $mM^{-1}cm^{-1}$ (Cherry et al., 1976). Ganglioside concentration was checked using a total sialic acid assay (Spiro, 1966).

Vesicle Preparation

Large bilayer vesicles (>2 μm in diameter) were prepared following a method similar to that described by Fahey and Webb (1978). An aliquot (0.4 ml) of a DMPC solution consisting of a concentration of 5 mg/ml in chloroform:methanol (2:1) was pipetted slowly on to the bottom of a 150 ml beaker. The solvent was allowed to dry under a stream of N_2. After complete evaporation 5 ml of double distilled H_2O was pipetted slowly onto the dried lipid film at a temperature above the phase transition temperature. After several hours the vesicles were collected. Vesicles containing the fluorescent probe DiI used in the photobleaching experiments were prepared by co-drying 1 ml of a 25mg/ml DMPC stock solution and 23 μl of a 3 mg/ml DiI stock solution in ethanol. Then 5 ml of $CHCl_3$:CH_3OH (2:1) was added to the dried lipid film. This resultant DMPC-DiI stock solution containing 0.2 mole % DiI was used as described above to make large bilayer vesicles. Large DPPC unilamellar vesicles of 900 Å diameter used in the pyrene experiments were prepared by fusing small sonicated vesicles at 4^oC as described elsewhere (Goins & Freire, 1985; Myers et al., 1984). Total phospholipid phosphorous was assayed using a modified Bartlett procedure (Marinetti, 1962).

Fluorescence Photobleaching Recovery

The instrumentation and data analysis used in these experiments have been described in more detail elsewhere (Barisas & Leuther, 1979; Leuther et al., 1981; Peacock & Barisas, 1983). A Coherent Radiation CR-2 argon ion laser provides excitation at 514 nm for CMTAA-eosin

labeled ganglioside G_{M1} and diI. A Zeiss universal microscope with vertical fluorescence illuminator and a 40X dry objective was used. Fluorescence was measured by an RCA C31034A photomultiplier tube coupled to a photon counter. The experiments in this paper were done using bleach times of 5 msec with a $1/e^2$ spot radius of 0.56 μm. Raw fluorescence recovery data were processed on line by a Nova 3/12 computer to yield the diffusion coefficient and fraction of mobile fluorescent molecules. Temperature was maintained by using a thermoelectrically controlled thermal stage. All the values presented in this thesis are the mean of at least 12 measurements.

Excimer Formation Experiments

Excimer formation experiments were performed by measuring the fluorescence emission spectrum of pyrene-PC incorporated into vesicles containing the desired amount of ganglioside G_{M1}. All fluorescence measurements were performed in a Perkin-Elmer LS-5 spectrofluorometer equipped with a red-sensitive photomultiplier and a thermostated cuvette holder connected to a Precision RDL 20 bath circulator. The temperature of the samples was monitored during the experiments with a Keithley digital thermometer. 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 respectively.

3. RESULTS

Ganglioside Binding to Cholera Toxin

The chemical structure of the fluorescent labeled ganglioside

G_{M1} (CMTAA-Eosin-G_{M1}) is shown in Figure III-1. In order to check the integrity and biological activity of the labeled ganglioside molecules, their ability to bind cholera toxin was examined. It has previously been reported (Goins & Freire, 1985; Mullin et al., 1976) that the binding of cholera toxin to its receptor, ganglioside G_{M1}, is accompanied by a blue shift in the tryptophan emission spectrum of the toxin. The tryptophan emission spectrum (excitation wavelength = 280 nm) was measured in the absence and in the presence of stoichiometric amounts of CMTAA-eosin ganglioside G_{M1}. As shown in Figure III-2, the emission maximum shifted from 347 nm to 341 nm after addition of the labeled ganglioside. This fluorescence change is similar to the one obtained with the native ganglioside and indicates that the tryptophan residues in the protein have become exposed to a more hydrophobic environment. It should be noted that neither the addition of phospholipid vesicles without ganglioside nor the addition of other gangliosides induced any changes in the fluorescence spectrum of the toxin molecule.

Ganglioside Lateral Diffusion as a Function of Temperature and Concentration

Before the photobleaching experiments, the phospholipid vesicles were observed by both phase contrast and epifluorescence microscopy to confirm that the labeled ganglioside had associated with the vesicles. The fact that the labeled ganglioside is quite mobile in the bilayer as judged by diffusion coefficients and recovery values provides further evidence that the ganglioside did incorporate into

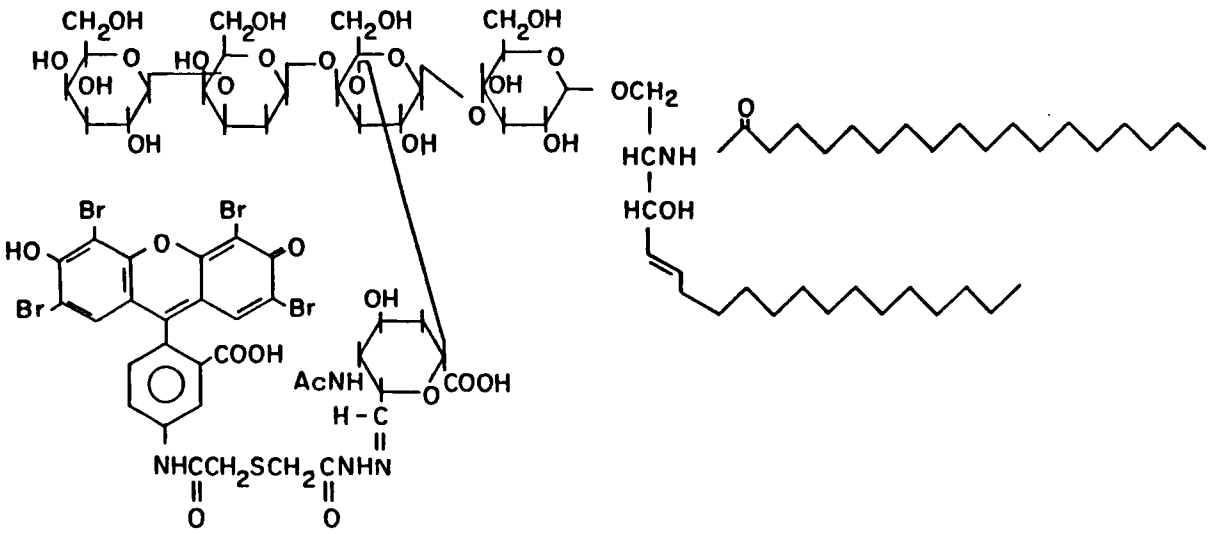

Figure III-1. Structure of 5 (((2-Carbohydrazino)methyl)thio)acetyl) amino eosin (CMTAA-eosin) modified ganglioside G_{M1}.

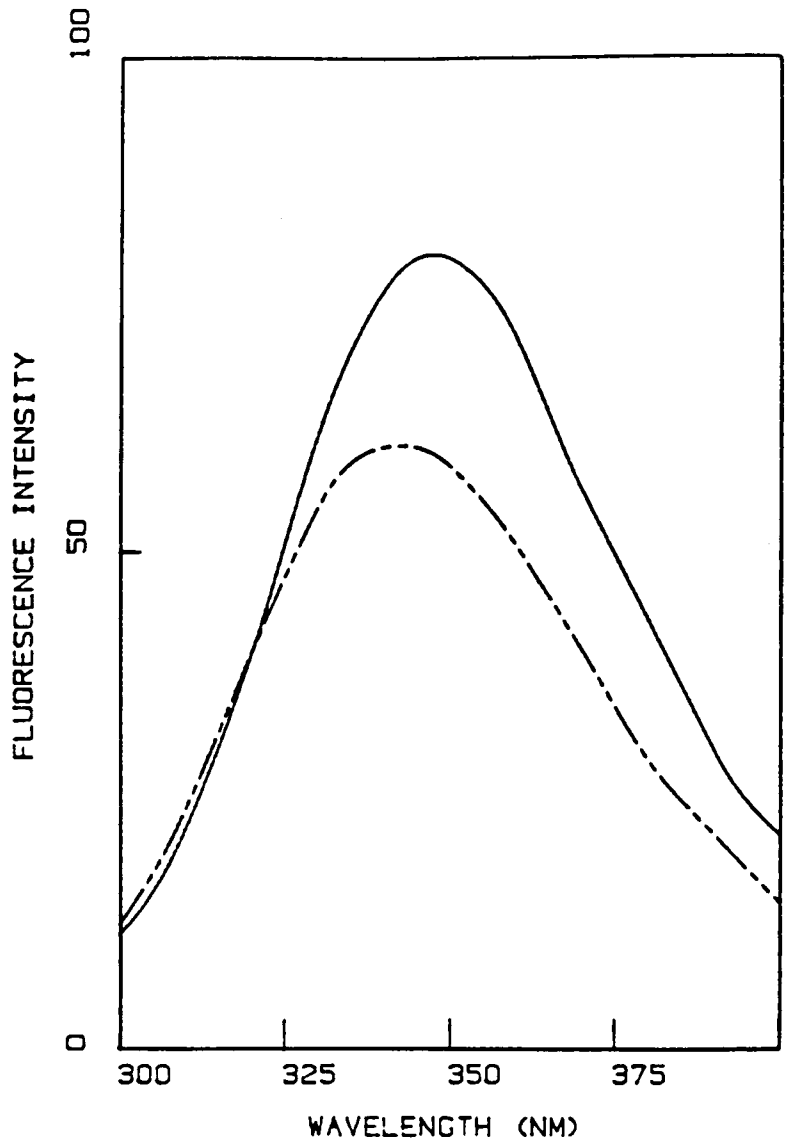

Figure III-2. Tryptophan emission spectrum of cholera toxin in the absence (solid line) or presence of stoichiometric amounts of CMTAA-eosin labeled ganglioside G_{M1} (dashed line).

the bilayer and had not just adhered to the vesicle surface. These observations agree with previous reports in which gangliosides have been shown to spontaneously incorporate within bilayers of model membrane systems (Sedlacek et al., 1976) and cells (Spiegel et al., 1984a).

The lateral diffusion of ganglioside G_{M1} incorporated into DMPC vesicles was measured as a function of temperature and mole fraction of ganglioside. The results are shown in Figure III-3. At all three ganglioside concentrations studied (1, 5 and 10 mole percent), the diffusion was dependent upon the gel-liquid crystalline state of the bilayer. At 25°C, slightly above the phase transition temperature for DMPC (T_m = 24.5°C) but still within the transition region, the diffusion coefficients were approximately 2.0 x 10^{-9} cm^2 sec^{-1} for all ganglioside containing samples. This value is significantly slower than the coefficient (D = 1.1 x $10^{-8}cm^2sec^{-1}$) found for the fluorescent lipid analogue diI in DMPC vesicles without ganglioside. This latter value is similar to the one previously obtained by Fahey and Webb (1978) under equivalent conditions. The addition of ganglioside also resulted in a reduced lateral diffusion coefficient (2.7 x 10^{-9} cm^2 sec^{-1}) of diI at this temperature. Previously, we (Goins & Freire, 1985) have found, using high sensitivity differential scanning calorimetry, that in the presence of ganglioside G_{M1} the heat capacity peak of the phospholipid becomes broader and skewed towards the high temperature end of the transition, and that this effect is probably due to a higher melting temperature of the ganglioside containing regions of the membrane. These results are consistent with

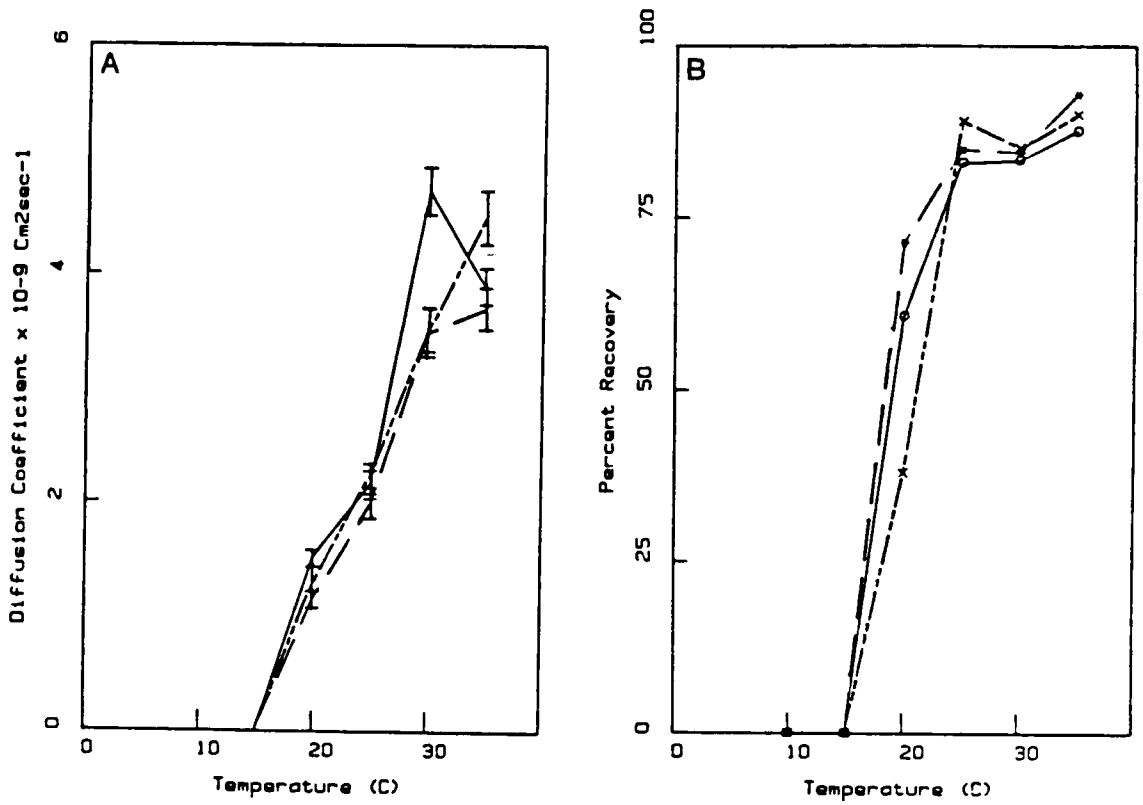

Figure III-3. Temperature dependence of the lateral mobility parameters for CMTAA-eosin labeled ganglioside G_{M1} incorporated into DMPC bilayers. Panel A: Lateral diffusion coefficient versus temperature for DMPC-G_{M1} bilayers at either 1% (——), 5% (— –) or 10% (---) total mole fraction of G_{M1}. In each case, the concentration of labeled ganglioside G_{M1} was constant. Panel B: Fluorescence percent recovery versus temperature for DMPC-G_{M1} bilayers at either 1% (0), 5% (*), or 10% (x) total G_{M1}.

the present observations and further suggest that ganglioside molecules only become fully mobile after the bulk of the phospholipid has melted. A 2-fold increase ($D = 4.7 \times 10^{-9} cm^2 sec^{-1}$) in the G_{M1} diffusion coefficient occurred when the DMPC vesicles containing ganglioside were incubated at 35°C which is above the phase transition temperature. This value is still slower than the one obtained for pure DMPC bilayers at this temperature ($D = 1.6 \times 10^{-8} cm^2 sec^{-1}$) using the fluorescent lipid analogue diI. This restricted movement on the part of the ganglioside in the fluid state of the bilayer is not due to the amount of total ganglioside present in the bilayer. This is further substantiated by the fact that the recovery in the fluorescence after bleaching (see Figure III-3B) is greater than 80% at and above the phase transition temperature for all three ganglioside concentrations. The lateral diffusion decreased only slightly by the presence of increasing amounts of unlabeled ganglioside. It appears that the addition of more ganglioside up to a mole fraction of 0.1 does not cause a measurable crowding or immobilization effect, thereby preventing the mobile labeled fraction from moving into the bleached area.

Effect of Calcium and Cholera Toxin on Ganglioside Lateral Mobility

The effect of calcium on the temperature dependence of the lateral diffusion of ganglioside G_{M1} in DMPC bilayers was also studied and the results are shown in Table III-1. The addition of up to 5 mM Ca^{2+} did not have a major effect on the measured diffusion

Table III-1. Effect of Ca^{2+} on the Lateral Diffusion Coefficient of Ganglioside G_{M1} in Gel or Fluid DMPC Bilayers

	1% G_{M1}*		10% G_{M1}	
Temperature (^{o}C)	Control	Ca^{2+}**	Control	Ca^{2+}
20	1.51 ± 0.074	1.81 ± 0.211	1.29 ± 0.068	2.18 ± 0.309
25	2.15 ± 0.122	2.36 ± 0.126	2.23 ± 0.106	2.46 ± 0.130
30	4.72 ± 0.206	3.36 ± 0.306	3.51 ± 0.190	3.56 ± 0.245

*Percent ganglioside depicts moles of ganglioside to total moles of lipid.

**5 mM Ca^{2+} was used throughout the experiment.

NOTE: All values are $x10^{-9}$ cm^2 s^{-1} and are represented as mean values ± standard errors.

coefficients for DMPC vesicles containing 1% and 10% ganglioside G_{M1} for all temperatures studied. Percent recoveries (data not shown) also remained the same even in the presence of calcium. Only the samples containing 1 mole % ganglioside showed a slight but statistically significant decrease in the lateral diffusion coefficient when Ca^{2+} was added above the phase transition temperature. Under all the remaining experimental conditions Ca^{2+} did not have a significant effect on the ganglioside mobility.

The substitution of the unlabeled ganglioside fraction for gangliosides G_{d1a} ($D = 3.44 \times 10^{-9} cm^2 sec^{-1}$) and G_{t1b} ($D = 3.83 \times 10^{-9} cm^2 sec^{-1}$) at a 10 mole percent concentration gave similar results. In the presence of Ca^{2+} the diffusion coefficients for G_{d1a} and G_{t1b} were $4.0 \times 10^{-9} cm^2 sec^{-1}$ and $3.33 \times 10^{-9} cm^2 sec^{-1}$ at 30°C, respectively. These gangliosides differ only in their number of sialic acid residues. It has been suggested that Ca^{2+} ions might have a crossbridging effect on gangliosides and that this effect may restrict the lateral movement of the ganglioside molecules (Sharom & Grant, 1978). From the photobleaching results it can be seen that the lateral mobility of gangliosides G_{M1}, Gd_{1a} and G_{t1b} is somewhat insensitive to the presence of Ca^{2+} ions.

The results obtained after the addition of stoichiometric amounts of cholera toxin to samples containing 1% or 10% ganglioside G_{M1} are shown in Figure III-4. In both cases the presence of the toxin caused a decrease in the measured lateral diffusion coefficient. However, this decrease in the lateral diffusion coefficient was not very large and was not accompanied by a decrease in the percent

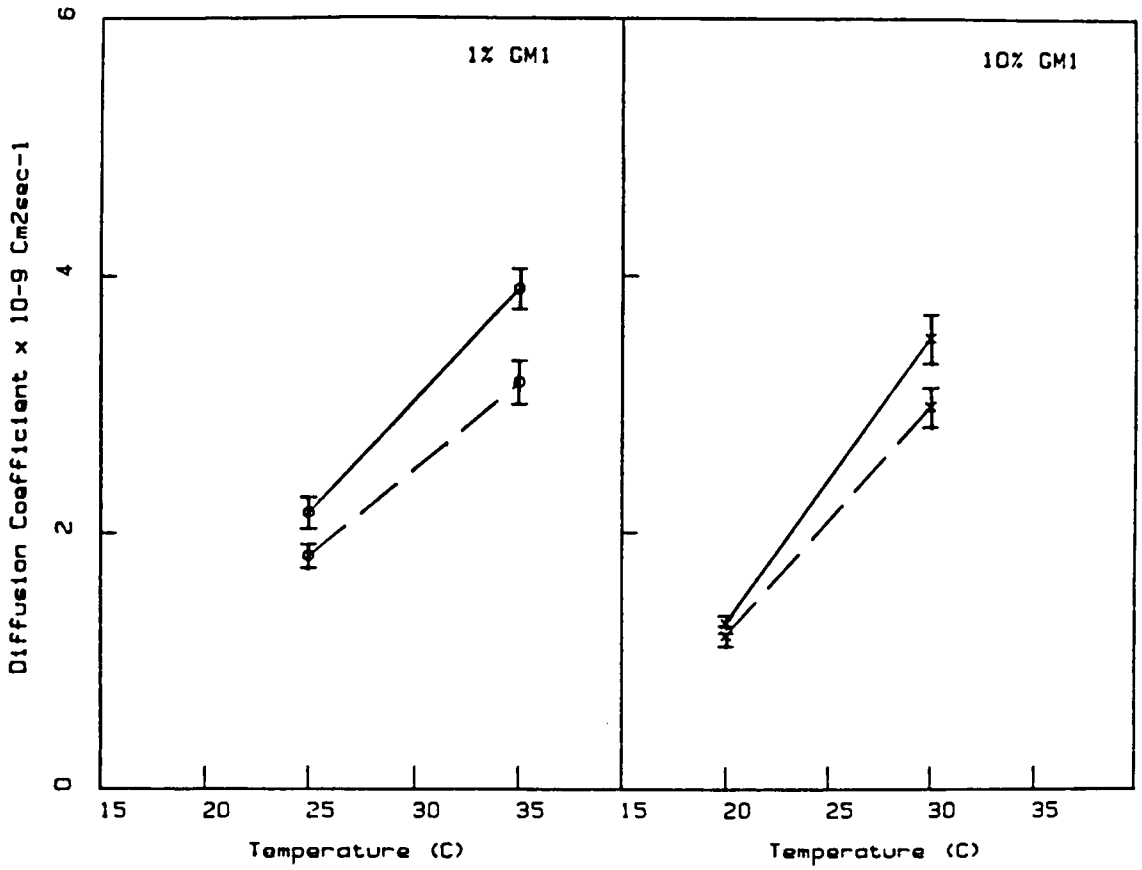

Figure III-4. Lateral diffusion coefficient vs. temperature for DMPC bilayers containing a total of either 1% G_{M1} (O) or 10% G_{M1} (x) in the presence (---) or absence (——) of stoichiometric amounts of cholera toxin. In both cases a constant amount of CMTAA-eosin labeled ganglioside G_{M1} was added.

recovery. Previously, we (Goins and Freire, 1985) have also observed a small decrease in the rate of pyrene excimer formation after the addition of cholera toxin to phospholipid vesicles containing ganglioside G_{M1}. Examination of the lipid vesicles under the fluorescence microscope did not reveal any evidence of patching or clustering after the addition of cholera toxin. These observations are in agreement with the results of Thompson et al. (1985) using freeze-etch electron microscopy to study the organization of ganglioside G_{M1} in phosphatidylcholine vesicles.

Excimer Formation Experiments

The technique of excimer formation by pyrene phospholipid derivatives has been used by several authors (Galla & Sackmann, 1974; Galla & Hartmann, 1980) to estimate lateral mobilities in artificial and natural membranes. For our present studies the excimer forming probe pyrene-PC was incorporated into large unilamellar DPPC vesicles containing different ganglioside G_{M1} mole fractions. The excimer/monomer ratios were measured as a function of temperature starting at 60°C and cooling the sample to temperatures below the phospholipid phase transition. The results of these experiments are illustrated in Figure III-5. The temperature dependence of the excimer/monomer ratios for pure DPPC vesicles is similar to that found by other authors and is characterized by an inflection at 41.5^{o}C, the transition temperature of DPPC. Above Tm the rate of excimer formation is diffusion controlled and the measured excimer/monomer ratios are proportional to the diffusion coefficient of the probe in

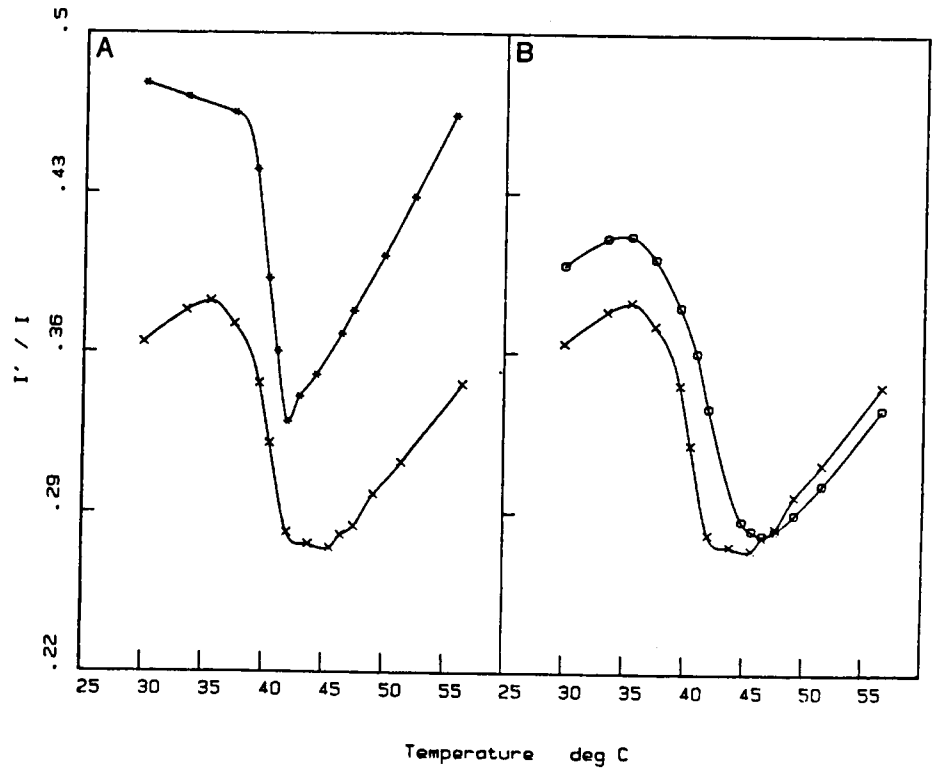

Figure III-5. The temperature dependence of excimer formation for pyrene PC incorporated into DPPC vesicles as a function of ganglioside G_{M1} and calcium concentration. Panel A: Excimer/monomer ratios vs. temperature of pyrene-PC incorporated into large unilamellar DPPC vesicles in the absence (*) or presence (x) of 10 mol % ganglioside G_{M1}. The lipid concentration was 3 μM and the amount of pyrene-PC was 3 mol % of the total lipid. Panel B: The temperature dependence on the excimer/monomer ratios of pyrene-PC incorporated into large unilamellar DPPC vesicles containing 10% G_{M1} in the absence (x) or presence (0) of 20 mM Ca^{2+}. Experimental conditions were the same as described in panel A.

the membrane (Galla & Hartmann, 1980). Above the phase transition temperature the addition of 10 mole % ganglioside G_{M1} causes a large decrease in the excimer/monomer ratio, consistent with a large restriction in the lateral mobility of the probe. At 55°C the excimer/monomer ratio is 0.46 for pure DPPC vesicles and decreases to 0.33 after incorporation of 10 mole % ganglioside. The addition of Ca^{2+} did not produce any significant difference in the excimer/monomer ratios above the phase transition temperature as indicated in Figure III-5B. These results are in agreement with the fluorescence recovery after photobleaching experiments in which no change in the measured diffusion coefficients were observed after the addition of Ca^{2+}.

4. DISCUSSION

Gangliosides have been implicated as cell surface membrane recognition sites for viruses, polypeptide hormones and bacterial toxins (Fishman & Brady, 1976). In particular, ganglioside G_{M1} has been identified as the receptor for cholera toxin (Fishman, 1982). The cholera toxin molecule is a multisubunit protein composed (Lai, 1980; Gill, 1976) of five binding (B) subunits (M = 11,000 per subunit) and an A subunit. This A subunit is composed of an active hydrophobic portion termed A1 (M = 21,000) linked via a disulfide bond to a smaller subunit, A2, (M = 5,000) which is responsible for holding the B and A1 subunits together. The binding subunits are able to bind ganglioside G_{M1} in a stoichiometric fashion and the intact toxin binds a maximum of five ganglioside molecules (Fishman et al., 1978). The association of cholera toxin to intact cells such as lymphocytes has

been shown to induce a massive redistribution and capping of the gangliosides molecules on the cell surface (Revesz & Greaves, 1975; Sedlacek, et al., 1976; Spiegel et al., 1984). The molecular mechanism of this process still remains unclear. The results presented in this communication were designed to elucidate the dynamic parameters of ganglioside diffusion in well-defined single component lipid bilayers, and to test whether or not the association of cholera toxin or the presence of Ca^{2+} has a significant effect on the distribution and mobility of gangliosides in a cell-free system.

Above the phase transition temperature of DMPC the lateral diffusion coefficient of CMTAA-ganglioside G_{M1} was approximately 4.7 x 10^{-9} $cm^2 sec^{-1}$. This value is in very close agreement with the lateral diffusion coefficient of 5.5 x 10^{-9} cm^2 sec^{-1} obtained by Reidler et al. (1978) for a fluorescein-labeled lyso ganglioside G_{M1} incorporated into 3T3 cells and also with the lateral diffusion coefficient obtained by Spiegel et al. (1984a) using rhodamine-labeled gangliosides incorporated into human fibroblasts. Our experiments indicate that the lateral diffusion coefficient measured by fluorescence photobleaching recovery is independent of the ganglioside mole fraction over the concentration range studied (1-10 mole percent), however this diffusion coefficient is about half of that of pure phospholipid vesicles under the same conditions. Excimer formation experiments were also consistent with a reduced lateral diffusion after incorporation of ganglioside G_{M1} in the lipid bilayer. This reduced lateral diffusion coefficient of the ganglioside is

expected on the basis of the bulkier ganglioside headgroup. The oligosaccharide headgroup of ganglioside G_{M1} has a molecular weight of 1016 compared to the molecular weight of 153 for phosphatidylcholine. The additional hydrodynamic drag will slow down the lateral diffusion of the ganglioside molecules. Previous electron spin resonance studies (Sharom & Grant, 1978) have also indicated an increased bilayer rigidity in the presence of glycosphingolipids.

The addition of cholera toxin in the phospholipid vesicles containing ganglioside G_{M1} did not produce any evidence of a massive redistribution of the fluorescence with the bilayer surface and decreased only slightly the lateral diffusion coefficient measured by fluorescence recovery after photobleaching. The lack of a distinguishable clustering in artificial bilayer systems after addition of cholera toxin is in agreement with recent results using freeze etch electron microscopy (Thompson et al. 1985) and should be contrasted with the massive redistribution observed in natural cell membranes. These results suggest that the driving force for capping is not simply the interaction between the ganglioside and the toxin molecule but that other elements present in intact cells, yet absent in phospholipid vesicles, must be considered.

The addition of Ca^{2+} did not cause a significant change in lateral diffusion above the phase transition temperature as indicated by the fluorescence photobleaching recovery experiments as well as the excimer formation experiments. These observations are in agreement with recent electrophoretic mobility and binding experiments by McDaniel and McLaughlin (1985). These authors concluded that the

intrinsic association constant of calcium with G_{M1} is quite low, less than 100 M^{-1}. The absence of a strong direct Ca^{2+}-ganglioside interaction accounts for the absence of a Ca^{2+} induced ganglioside clustering in the fluid phase of the bilayer. Recent calorimetric experiments (Masserini & Freire, 1986) using homogeneous chain length gangliosides indicate that the Ca^{2+} induced solid phase immiscibility observed in calorimetric scans of ganglioside-phosphatidylcholine mixtures (Goins & Freire, 1985; Myers et al., 1984) is ganglioside chain length dependent and is probably triggered by a tightening of the phosphatidylcholine bilayer matrix followed by ganglioside passive exclusion from the phosphatidylcholine rich phase, rather than by active crossbridging of ganglioside molecules by Ca^{2+}.

PART III

REFERENCES

REFERENCES

Barisas, B. G., and Leuther, M. D. (1979) Biophys. Chem. 10: 221-229.

Behr, J. P., and Lehn, J. M. (1973) FEBS Lett. 31: 297-300.

Cherry, R. J., Cogoli, A., Oppliger, M., Schneider, G., and Semenza, G. (1976) Biochemistry 15: 3653-3656.

Cherry, R. J., Nigg, E. A., and Beddard, G. S. (1980) Proc. Natl. Acad. Sci. U.S.A. 77: 5899-5903.

Fahey, P. F., and Webb, W. W. (1978) Biochemistry 17: 3046-3053.

Felgner, P. L., Freire, E., Barenholz, Y., and Thompson, T. E. (1981) Biochemistry 20: 2168-2172.

Fishman, P. H. (1982) J. Membr. Biol. 69: 85-97.

Fishman, P. H., Moss, J., and Osborne, J. C., Jr. (1978) Biochemistry 17: 711-716.

Fishman, P. H., and Brady, R. O. (1976) Science 194: 906-915.

Galla, H. J., and Hartmann, W. (1980) Chem. Phys. Lipids 27: 199-219.

Galla, H. J., and Sackmann, E. (1974) Biochim. Biophys. Acta 339: 103-115.

Gill, D. M. (1976) Biochemistry 15: 1242-1248.

Goins, B., and Freire, E. (1985) Biochemistry 24: 1791-1797.

Hakomori, S. (1981) Annu. Rev. Biochem. 50: 733-764.

Hansson, H. A., Holmgren, J., and Svennerholm, L. (1977) Proc. Natl. Acad. Sci. U.S.A. 74: 3782-3786.

Jaques, L. W., Brown, E. B., Barrett, J. M., Brey, W. S., Jr., and Weltner, W., Jr. (1977) J. Biol. Chem. 252: 4533-4538.

Jeng, I. (1983) in Neural Membranes Experimental and Clinical Neuroscience (Sun, G. Y., Bazan, N., Wu, J. Y., Procellah, G. and Sun, A. Y., Eds.) pp. 513-526, Humana Press, Inc., New York.

Kundu, S. K. (1981) Methods Enzymol. 72: 185-204.

Lai, C. Y. (1980) CRC Crit. Rev. Biochem. 8: 171-206.

Ledeen, R. W. (1978) J. Supramol. Struct. 8: 1-17.

Leuther, M. D., Peacock, J. S., Krakauer, H., and Barisas, B. G. (1981) J. Immunol. 127: 893-899.

Marinetti, G. V. (1962) J. Lipid Res. 3: 1-20.

Masserini, M., and Freire, E. (1985) Biochemistry 25: 1043-1049.

McDaniel, R. V., and McLaughlin, S. (1985) Biophys. J. 47: 424a.

Mullin, B. R., Aloj, S. M., Fishman, P. H., Lee, G., Kohn, L. D., and Brady, R. O. (1976) Proc. Natl. Acad. Sci. U.S.A. 73: 1679-1683.

Myers, M., Wortman, C., and Freire, E. (1984) Biochemistry 23: 1442-1448.

Peacock, J. S., and Barisas, B. G. (1983) Biochemistry 131: 2924-2929.

Rahmann, H., Probst, W., and Muhleisen, M. (1982) Japan J. Exp. Med. 52: 275-286.

Reidler, J., Eldridge, C., Schlessinger, Y., Elson, E., and Wiegandt, H. (1978) J. Supramol. Struct. Suppl. 2: 306a.

Revesz, T., and Greaves, M. (1975) Nature 257: 103-106.

Sedlacek, H. H., Stark, J., Seiler, F. R., Ziegler, W., and Wiegandt, H. (1976) FEBS Lett. 61: 272-276.

Sharom, F. J., and Grant, C. W. M. (1978) Biochim. Biophys. Acta 507: 280-293.

Spiegel, S., Schlessinger, J., and Fishman, P. H. (1984) J. Cell Biol. 99: 699-704.

Spiegel, S., Kassiso, S., Wilchek, M., and Fishman, P. H. (1984) J. Cell Biol. 99: 1575-1581.

Spiro, R. G. (1966) Methods Enzymol. 8: 1-52.

Steck, T. L., and Dawson, G. (1974) J. Biol. Chem. 249: 2135-2142.

Tettamanti, G., Preti, A., Cestaro, B., Venerando, B., Lombardo, A., Ghidoni, R., and Sonnino, S. (1979) in Structure and Function of Gangliosides (Svennerholm, L., Mandel, P., Dreyfus, H. and Urban, P. F., Eds.) pp. 263-281, Plenum Press, New York.

Thompson, T. E., Johnson, M., Alietta, M., Brown, R. E., and Tillack, T. W. (1985) Biochim. Biophys. Acta 817: 229-237.

Tuszynski, G. P., Knight, L., Piperno, J. R., and Walsh, P. N. (1980) Anal. Biochem. 106: 118-122.

PART IV

THERMAL STABILITY AND INTERSUBUNIT INTERACTIONS OF CHOLERA TOXIN IN SOLUTION AND IN ASSOCIATION TO ITS CELL SURFACE RECEPTOR, GANGLIOSIDE G_{M1}

THERMAL STABILITY AND INTERSUBUNIT INTERACTIONS OF CHOLERA TOXIN IN SOLUTION AND IN ASSOCIATION TO ITS CELL SURFACE RECEPTOR, GANGLIOSIDE G_{M1}

1. INTRODUCTION

The molecular mechanism by which cholera toxin interacts with eucaryotic cells has been of interest to many researchers for many years [For a review see van Heyningen and Seal, 1983]. Cholera toxin is a multisubunit protein consisting of five identical binding (B) subunits (M = 11,500) forming a pentameric ring which supports the A subunit (Gill, 1976). Treatment of the A subunit with reducing reagents produces two subunits: an ADP-ribosylating protein termed A1 (M = 21,000) which activates adenylate cyclase and a small protein, A2 (M = 6,000) which plays a structural role in holding the A1 and B subunits together.

At the present time, the mechanism of action of cholera toxin is not completely understood at the molecular level. The interaction of cholera toxin with the cell surface occurs when the B subunit recognizes and binds, with high affinity and specificity, to ganglioside G_{M1}. The association of the cholera toxin B subunit to ganglioside G_{M1} is believed to cause a conformational change in the protein that facilitates the exposure and subsequent penetration of the A subunit into the membrane. Evidence for this conformational change has been derived from fluorescence studies using cholera toxin incubated with either ganglioside G_{M1} (Mullin et al., 1976) or the oligosaccharide moiety of G_{M1} (Fishman et al., 1978) and NMR studies

(Sillerud et al., 1981). Various techniques including photoaffinity labeling (Wisnieski & Bramhall, 1981; Tomasi & Montecucco, 1981; Tomasi et al., 1982), hydrodynamic studies (Dwyer & Bloomfield, 1982) and differential scanning calorimetry (Goins & Freire, 1985) have provided indirect evidence that upon association of cholera toxin with lipid vesicles containing gangioside G_{M1}, the A subunit penetrates into the hydrophobic core the lipid bilayer. More recently, three-dimensional structural studies using electron crystallography at 15Å resolution (Ludwig et al., 1986; Ribi & Kornberg, 1987) have revealed that the A subunit initially does insert into the lipid milieu and that upon reduction of the A subunit there is a further penetration of the enzymatically active A1 subunit into the membrane. The A1 subunit is very hydrophobic in nature and can be reconstituted into lipid vesicles even in the absence of ganglioside (Goins & Freire, 1985).

In Part IV of this thesis, the thermal stability of the intact cholera toxin and cholera toxin subunits has been determined using high sensitivity differential scanning calorimetry (DSC) and differential solubility thermal gel analysis (Rigell & Freire, 1987). Also the energtics of association of cholera toxin to ganglioside G_{M1} has been measured by high sensitivity isothermal titration calorimetry. These studies have permitted us to characterize the magnitude of the thermodynamic parameters that determine the stability of this multisubunit protein, to evaluate the cooperative interactions between subunits and to assess how these parameters depend on the

association of the toxin molecule to its cell surface receptor, ganglioside G_{M1}.

2. MATERIALS AND METHODS

Protein

Intact cholera toxin and cholera toxin B subunit were purchased from Sigma (St. Louis, MO). The proteins were supplied as lyophilized powders and were reconstituted with distilled water to give the appropriate concentration of protein in 50mM Tris-HCl, 0.2M NaCl, 3mM NaN_3, and 1mM Na_2EDTA, pH 7.5. The purity of the protein samples was checked by SDS gel electrophoresis using the procedure described by Laemmli (1970). EDTA-free protein samples for calorimetric and differential solubility gel analysis experiments were prepared by dialyzing the toxin stock solution against 4000 volumes of buffer containing no EDTA overnight at 4^oC. Protein samples for the isothermal titration experiments were prepared by dialyzing overnight at 4^oC a toxin stock solution against 4000 volumes of 10mM sodium phosphate buffer containing 0.02% NaN_3, pH 7.4. All protein stocks and experimental protein samples were assayed for protein concentration by the method of Lowry (1951).

Liposome Preparation

Small unilamellar vesicles were prepared according to Schullery et al. (1980). Egg phosphatidylcholine (egg PC), purchased from Avanti Polar Lipids (Birmingham, AL) was N_2 dried from a chloroform solution and lyophilized overnight. The dried lipid was resuspended

in 50 mM Tris-HCl, 0.2M NaCl and 3 mM NaN_3, pH 7.5 to give a lipid concentration of 9 mM. The lipid suspension was then sonicated until clear using a bath sonicator (Model G112 SP1G, Laboratory Supplies, Hicksville, NY) and centrifuged at 15,000 x g for 1 hour to remove residual multilamellar vesicles. The upper two-thirds of the supernatant following centrifugation was used in the differential solubility thermal gel analysis experiments. To avoid fusion of the vesicles prior to use, they were kept in a thermostated block set for 30°C. Vesicles were prepared fresh for each experiment.

Ganglioside G_{M1}

The ganglioside G_{M1} used in these experiments was prepared by enzymatic cleavage of sialic acid residues from di- and trisialogangliosides using the procedure outlined in Part I of this thesis. The di- and trisialogangliosides used for the isolation procedure were obtained from the Folch extract of bovine brain (Avanti Biochemicals, Birmingham, AL) using the procedure described by Myers et al. (1984). Stock ganglioside solutions were checked by the total sialic acid determination using the resorcinol method described by Spiro (1966). Incorporation of ganglioside into phospholipid vesicles was performed by adding a given amount of G_{M1} stock solution in Tris buffer and incubating the mixture above the phospholipid phase transition temperature for 90 minutes. Following incubation, the vesicles were used immediately for differential solubility thermal gel analysis experiments. This allows for an asymmetric distribution of ganglioside in which ganglioside is found only on the outer leaflet of

the vesicles (Felgner et al., 1981). Ganglioside concentrations were 10 mol % of the total lipid concentration for the differential solubility gel analysis experiments.

Differential Scanning Calorimetry

All calorimetric scans were performed using a Microcal MC-2 differential scanning calorimeter. The calorimetric unit was interfaced to an IBM PC microcomputer using an A/D converter board (Data Translation DT-2801) for automatic data collection and analysis. The protein concentration of each sample used for the calorimetric experiments was approximately 2 mg/ml. The sample (1.3 ml) was injected into the sample cell and the same amount of buffer was used in the reference cell. Samples were heated at a rate of 60°C/hr. Cholera toxin samples were degassed 15 minutes at room temperature prior to scanning. To prepare samples containing cholera toxin and ganglioside G_{M1}, stock solutions were degassed separately for 15 minutes after which time aliquots of each were added to the desired volume of buffer and incubated under vacuum an additional 5 minutes at room temperature prior to scanning.

Differential Solubility Thermal Gel Analysis

The thermotropic behavior of cholera toxin alone and in the presence of ganglioside G_{M1} was also examined using a variation of the technique of differential solubility thermal gel analysis (Rigell & Freire, 1987). This technique allows one to investigate the thermal denaturation of complex multisubunit proteins by examining solubility changes in individual subunits, induced during the heating of the

protein. Briefly, a separate aliquot of cholera toxin (100 µl of a 1 mg/ml stock solution dissolved in cholera toxin buffer) was prepared for each temperature point of the thermal denaturation profile. In addition, each sample contained either 0.09 µmoles micellar ganglioside G_{M1}, 0.9 µmoles egg PC vesicles, 0.9 µmoles egg PC vesicles containing 10 mol % ganglioside G_{M1}, or buffer. A constant volume of 200 µl was used in all experiments. When present, the concentration of ganglioside G_{M1} in the samples corresponded to a 76 fold excess in order to ensure complete saturation of the toxin. For these experiments, a programmable scanning water bath (Neslab RTE-8 and temperature programmer ETP3) was used. The temperature of the bath was continuously monitored by a thermistor connected to a Hewlett-Packard logging multimeter. The thermistor was submerged in a tube filled with 200 µl of cholera toxin buffer. At each designated temperature, the appropriate samples were placed for 1 minute in the bath, removed and placed on ice for 10 minutes. Immediately after, each sample was centrifuged at 1500 rpm for 90 seconds to remove any water condensation from the sides of the test tubes and then transferred to a microfuge tube for centrifugation at 15,000 xg for 15 minutes. Iodine and resorcinol detection of thin-layer chromatograms (Kates, 1972) developed in $CHCl_3$:CH_3OH:H_2O (65:25:4) were utilized to monitor the lipid and ganglioside content respectively of the supernatant and pellet fractions. Under these conditions, both the lipid and ganglioside remained in the supernatant fraction of samples heated up to 100°C, and only free A subunit (see results) was

pelleted, thus allowing one to quantitate the amount of A subunit uptake by ganglioside micelles or phospholipid vesicles.

Since ganglioside G_{M1} and lipid both interfere with SDS-gel electrophoresis (Gill, 1977), they were removed from the supernatant fractions of each sample prior to electrophoresis by incubating with 0.7 ml cold acetone on ice for 15 minutes. The samples were centrifuged at 15,000 xg for 15 minutes and the acetone containing-supernatant discarded. To remove any residual lipid, the pellet was washed with 0.7 ml cold chloroform:methanol (2:1). Following a 15 minute incubation, the chloroform methanol mixture was discarded and the pellet dried under vacuum for 30 minutes. The pellet was then reconstituted with 200 µl of cholera toxin buffer and processed for electrophoresis.

Usually the sample was incubated overnight with 40 µl of a 5x concentrated electrophoresis sample buffer containing 15% 0.4 M Tris-HCl pH 7.4, 50% sucrose, 4% SDS and bromophenol blue, as the tracking dye. A 30 µl aliquot of each sample (approximately 12.5 µg of protein) was run on a 15% polyacrylamide gel overlaid with a 5% polyacrylamide stacking gel following the procedure of Laemmli (1970). Electrophoresis was run for two hours at 60V (constant) and then four hours at 150V (constant). Gels were stained in methanol:acetic acid:H_2O (5:4:1) containing 0.2% Coomassie brilliant blue and subsequently destained in water:methanol:acetic acid (8:1:1). Densitometry of the destained gels was performed using a LKB laser densitometer (2202 Ultrascan) interfaced to an IBM PC microcomputer using a Data Translation (DT-2805) A/D converter board. The gels were

scanned from bottom (lower molecular weight species) to top (high molecular weight species). Data collection and subsequent data manipulation to obtain normalized peak intensities and areas was performed using software developed in this laboratory.

High Sensitivity Isothermal Titration Calorimetry

High sensitivity isothermal titration calorimetry was performed with a differential calorimetric titration module designed and built in Dr. Freire's laboratory. This instrument operates under computer control and has a sensitivity better than 10 ncal/sec thus allowing a direct measurement of the energetics of biochemical reactions using nanomolar quantities of material (Myers et al., 1987).

These experiments were performed at 20°C. The sample cell contained in 1 ml total volume a 1 mM ganglioside G_{M1} solution in 10 mM sodium phosphate containing 0.02% NaN_3, pH 7.4. The titration experiments consisted of a sequence of 30 μl injections at predetermined time intervals, each containing either 2 nmoles of cholera toxin or 3 nmoles of B subunit pentamer. The calorimetric reference cell contained 1 ml of 10 mM sodium phosphate buffer containing 0.02% NaN_3, pH 7.4 and the same buffer was loaded into the injection device. During the experiment there was continuous stirring of the sample and reference cell contents. The differential heat effects between both cells were measured and stored for further analysis using software developed in Dr. Freire's laboratory.

3. RESULTS

Thermal Stability of Cholera Toxin

The excess heat capacity versus temperature for intact cholera toxin and isolated pentameric B subunits are shown in Figure IV-1. As shown in Figure IV-1A the calorimetric scan of intact cholera toxin is characterized first by the appearance of a peak centered at 51°C with an enthalpy change of 90 Kcal/mole. This peak is followed by an exothermic process at approximately 70°C. Visual inspection of the sample indicates that this exotherm is associated with the appearance of a white precipitate. If intact cholera toxin is scanned only up to the temperature at which the precipitate begins to appear (Figure IV-1B) and then cooled and rescanned the calorimetric profile shown in Figure IV-1C is observed. This scan is characterized by a single peak centered at 74°C and an enthalpy change of 520 Kcal/mole. This calorimetric scan is similar to the one obtained with isolated B subunit pentamers (Figure IV-1D) and indicates that the peak at 74°C corresponds to the denaturation of the binding subunits in the intact toxin.

Gel electrophoresis analysis of the precipitate and the supernatant obtained after centrifugation of a sample scanned up to 70°C revealed that the A subunit was present exclusively in the precipitate and that the supernatant contained only the B subunit. These experiments indicate that the A subunit denatures at 51°C but it is only released from the toxin molecule at the onset of the B subunit

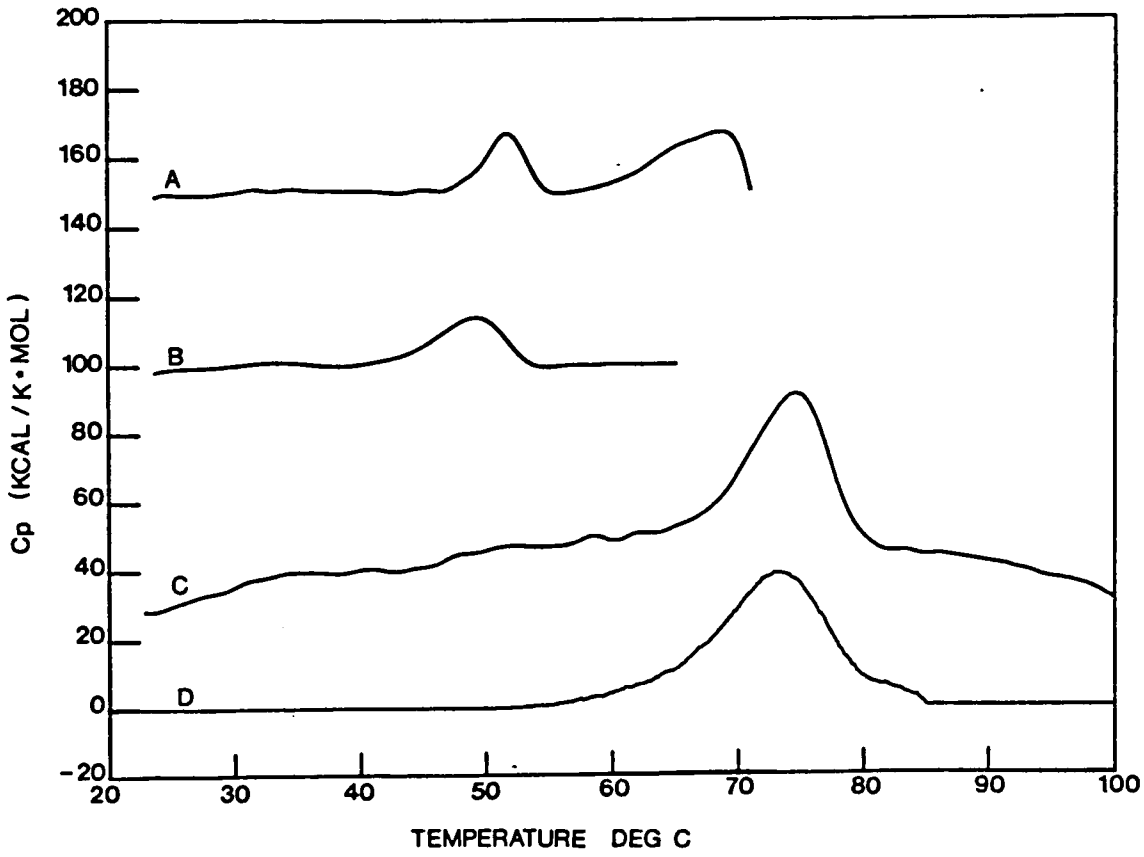

Figure IV-1. Heat capacity profiles for either cholera toxin or isolated B subunit pentamer free in aqueous solution. Curve A depicts the excess heat capacity function versus temperature plot of cholera toxin showing the initial portion of the exothermic effect at 70°C. Curve B depicts a scan of a different sample of toxin stopped immediately prior to the exotherm. Curve C shows a rescan of the sample in B. Curve D shows the calorimetric heat capacity profile for the isolated B subunit pentamer.

pentamer unfolding. Due to its hydrophobic nature, the A subunit precipitates after it dissociates from the toxin molecule.

Thermotropic Behavior of Cholera Toxin in the Presence of Ganglioside G_{M1}

The conformational stability of intact cholera toxin when bound to ganglioside G_{M1} was also studied by high sensitivity differential scanning calorimetry. As shown in Figure IV-2, the unfolding transition of the B subunit pentamer of intact cholera toxin centered at 74°C (trace A) is shifted to higher temperatures and broadened after the addition of ganglioside G_{M1} micelles at low toxin:ganglioside molar ratios. It should be noted that the concentration of ganglioside present in all calorimetric samples was above the critical micellar concentration of 1×10^{-6} M (Masserini & Freire, 1987). The broad multiple peaks evident at low protein/ gangioside mole ratios give rise to a single and progressively sharper peak centered at 95°C at increasing ganglioside concentrations. The upward shift of 20°C in the transition temperature denotes a stabilization of the B subunit native conformation in the presence of ganglioside G_{M1}.

At saturating concentrations of ganglioside, the heat capacity profile is noticeably sharper than in the absence of ganglioside indicating an increase in the cooperative interactions between individual monomers within the B subunit pentamer. The cooperative unit (n), a measure of cooperativity derived from the ratio of the van't Hoff enthalpy to calorimetric enthalpy ($\Delta H_{VH}/\Delta Hcal$), was

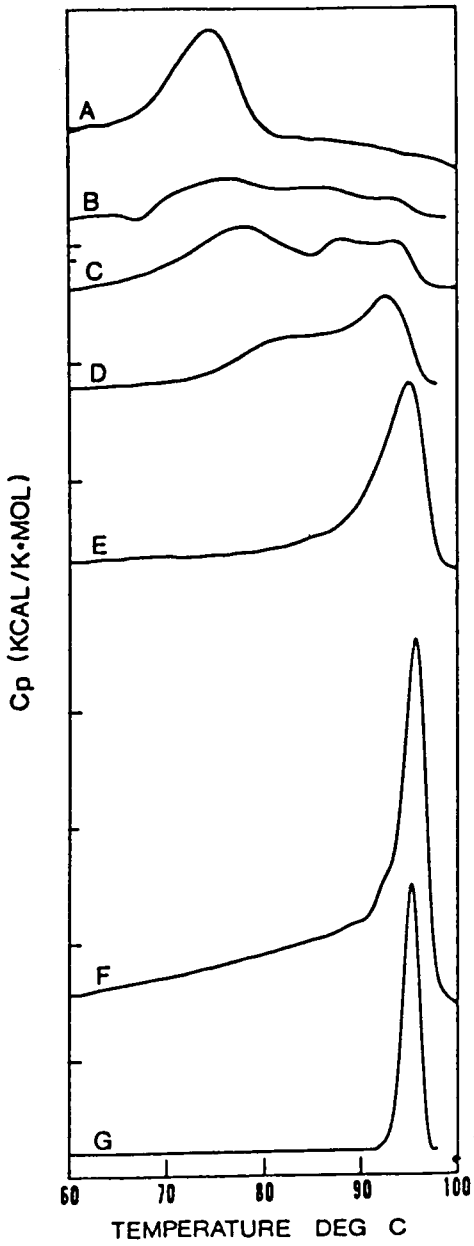

Figure IV-2. Enlargement of the B subunit transition region from heat capacity profiles of intact cholera toxin incubated with increasing amounts of ganglioside G_{M1}. The mole ratios of ganglioside G_{M1} to intact toxin are: 0 (A), 0.46:1 (B), 1.5:1 (C), 3.7:1 (D), 7:1 (E), 23:1 (F), and 77:1 (G). Scans are offset for presentation purposes. Each interval on the y-axis represents 50 Kcal/K·mol.

determined in these experiments. When the calorimetric enthalpies were normalized to the concentration of intact toxin present in the sample, a value of 0.169 was calculated in the absence of ganglioside G_{M1}, whereas at saturation (trace G) a value of 0.932 was determined. These results indicate that in the absence of ganglioside the individual monomers of the B subunit pentamer unfold independently of one another; but that at saturation, the B subunit pentamer unfolds as a single unit. Similar thermotropic behavior is exhibited by the B subunit pentamer alone when scanned in the presence of ganglioside G_{M1} (unpublished observation; Dalziel et al., 1984).

Also, it should be noted that the exothermic effect, observed in the absence of ganglioside is absent in the calorimetric scans obtained in the presence of ganglioside. These results together with gel electrophoresis analysis (see below) indicate that the A subunit does not precipitate if the toxin is bound to ganglioside G_{M1}, suggesting that the A subunit or at least part of the A subunit partitions into the hydrophobic interior of the ganglioside micelle.

Figure IV-3 shows the excess heat capacity function associated with the thermal denaturation of the A subunit. These curves were obtained from calorimetric scans of intact cholera toxin in the presence of increasing concentrations of ganglioside G_{M1}. As shown in Figure IV-3, saturating concentrations of ganglioside G_{M1} have only a very small effect on the A subunit transition even though the transition of the B subunit has been shifted twenty degrees to higher temperatures. Under these conditions the A subunit transition is shifted only one degree. These results indicate that the interactions

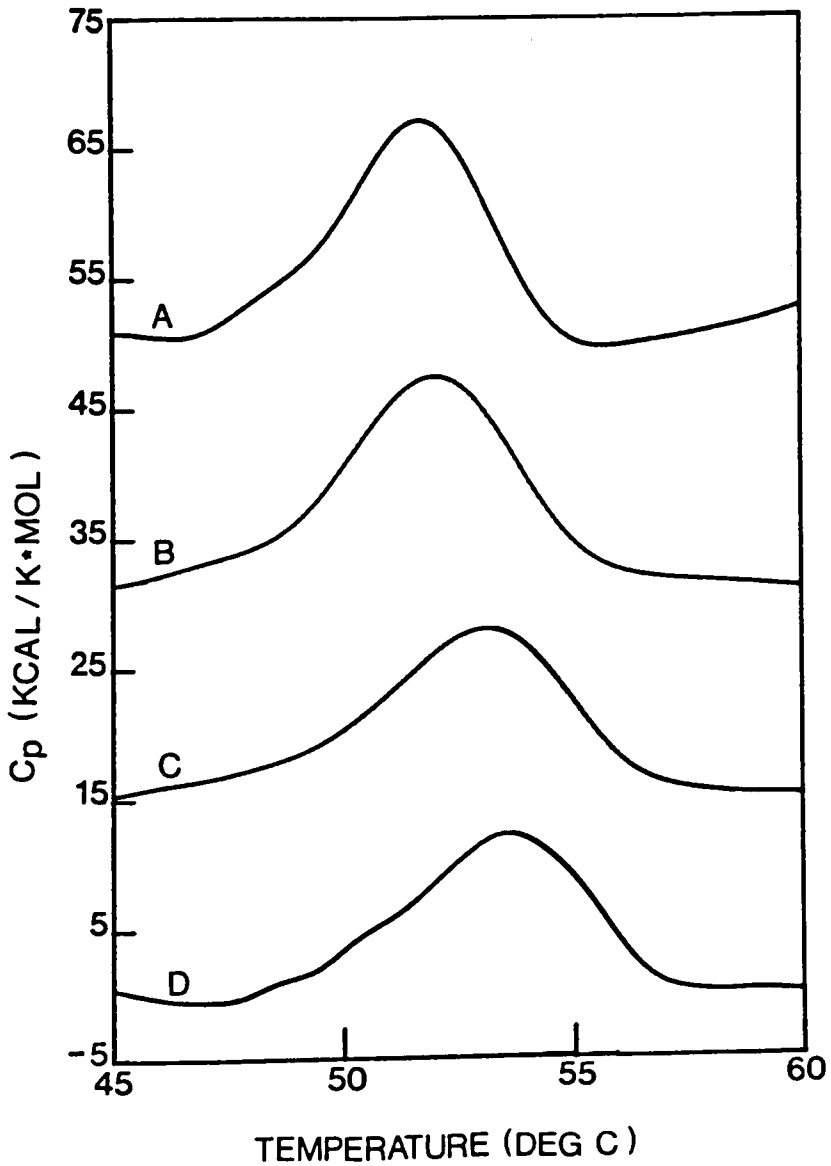

Figure IV-3. Enlargement of the A subunit transition region from calorimetric scans of intact cholera toxin scanned with increasing concentrations of ganglioside G_{M1}. The mole ratios of ganglioside G_{M1} to intact toxin are: 0 (A), 0.46:1 (B), 9:1 (C), 23:1 (D). Scans are offset for presentation purposes.

between the A and B subunits are probably not affected by the association of ganglioside G_{M1} to the B subunit suggesting that these interactions are only of a very local nature. This conclusion is also consistent with the observation that the unfolding profile of the B subunit is not affected by the presence or absence of the A subunit in the toxin molecule. This should be contrasted with the dramatic enhancement of the inter-B-subunit cooperative interactions induced by ganglioside G_{M1}.

The transition temperatures calculated from the maximum in the heat capacity function for the thermal transition of the A and B components of cholera toxin upon addition of ganglioside G_{M1} are shown in Figure IV-4. As discussed before, the association of ganglioside G_{M1} to cholera toxin has a profound effect on the B subunit transition temperature but only a slight affect on the A subunit transition temperature. The transition temperature of the B subunit increases monotonically upon the addition of ganglioside G_{M1} up to the concentration at which saturation is reached at a G_{M1}:protein ratio of 5:1.

Reversibility of Thermal Transitions

The thermal denaturation of the cholera toxin A subunit is an irreversible process as demonstrated by the disappearance of the transition at 51°C upon rescanning of the same sample (Figure IV-1B and C). On the contrary, the thermal denaturation of the B subunit pentamer is a reversible process, provided that the protein is not kept at post-denaturational temperatures for long periods of time.

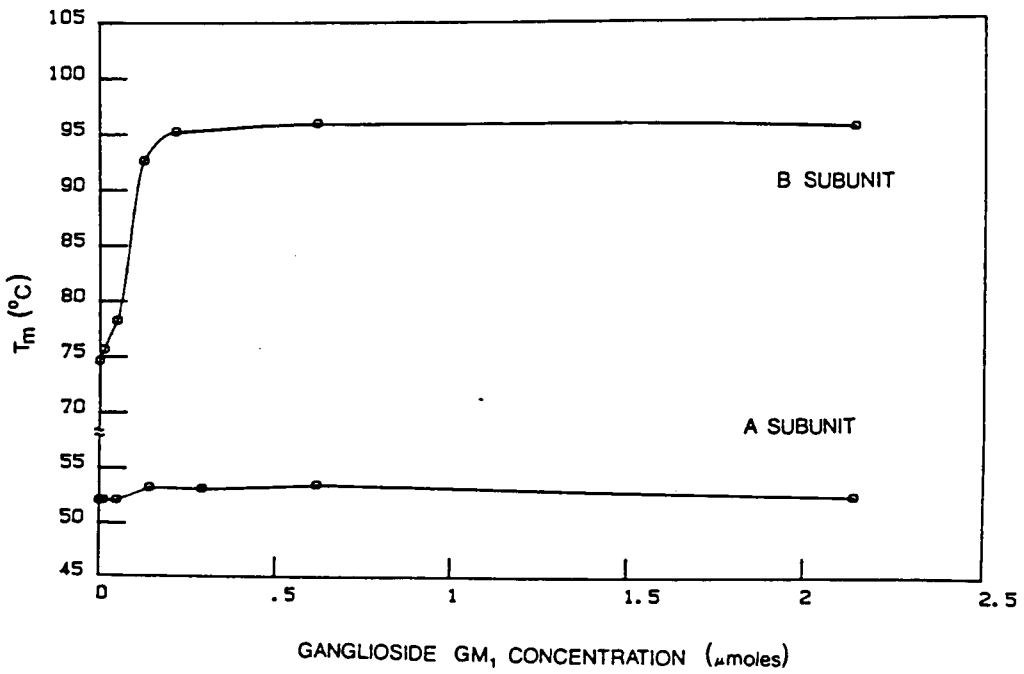

Figure IV-4. Dependence of the transition temperature on the concentration of ganglioside G_{M1} for the A and B subunit transitions of cholera toxin.

This behavior is illustrated in Figure IV-5 for successive calorimetric scans of isolated B subunit pentamers. As depicted in Figure IV-5, the area under the heat capacity curve decreases progressively for each successive scan. In fact, if the areas are plotted as a function of the time that the sample has been kept above the transition region an exponential decay is obtained, as shown in Figure IV-6. This exponential decay is characterized by a relaxation time of 10 minutes.

The reversibility of the isolated B subunit pentamer transition in the presence of excess ganglioside G_{M1} (B subunit/G_{M1} = 1/50) was also studied and the results are shown in Figure IV-6 (dashed line). An exponential decay characterized by a relaxation time of 2 minutes was obtained under these conditions. The B subunit pentamer transition of intact cholera toxin in the absence and presence of ganglioside G_{M1} also shows a similar reversible behavior (data not shown).

These calorimetric results suggest the following transition mechanism for the cholera toxin B subunit:

$$F \rightleftharpoons U \rightarrow P$$

According to the above mechanism the B subunit pentamer exists in rapid equilibrium between the folded and unfolded states. This equilibrium is followed by an irreversible process, P, which is triggered when the unfolded state becomes populated. The rate of transformation of the irreversible denatured species, P, is temperature dependent and characterized by an activation enthalpy of

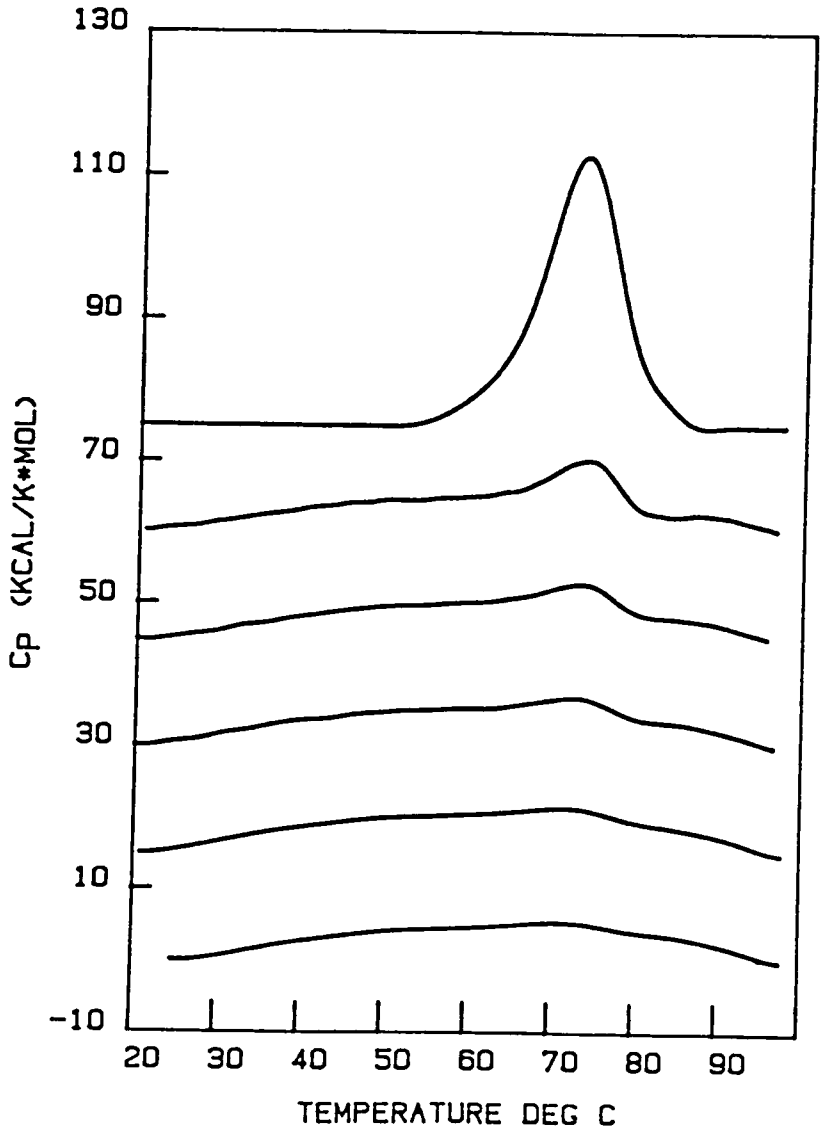

Figure IV-5. A series of consecutive DSC scans of isolated B subunit pentamer. Each successive scan was performed after immediately cooling the sample in the calorimeter and then rescanning under the same conditions. Scans are offset in the Y axis for presentation purposes.

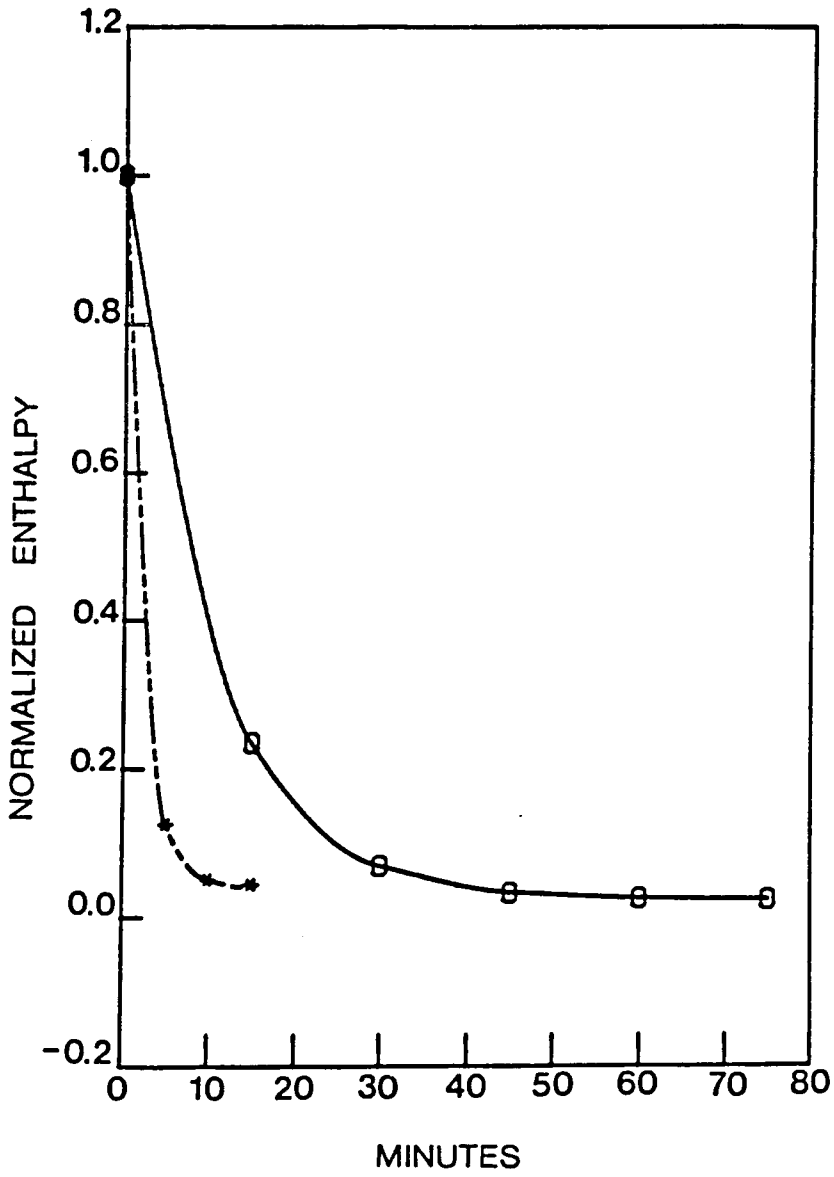

Figure IV-6. Dependence of the degree of reversibility of the B subunit unfolding transition on the time spent by the sample above the transition temperature in the absence (solid line) or presence of excess ganglioside G_{M1} (dashed line). Enthalpies were normalized to the enthalpy of the first scan in the series. Raw data points were fitted using a single exponential decay curve.

25 Kcal/mole. This mechanism appears to be of a general nature and obeyed by small as well as large proteins including those lacking disulfide bridges. For example, staphylococcus nuclease also exhibits a post-denaturational irreversible step characterized by a relaxation time of ~60 min at pH 6.3 in which the unfolding transition is centered at 52°C. Similarly, the same phenomenon occurs with ribonuclease A, even though in this case the relaxation time is approximately four hours (unpublished data from Dr. Freire's laboratory).

Differential Solubility Thermal Gel Analysis

In order to complement the DSC data, the interactions of the cholera toxin subunits among themselves and with ganglioside G_{M1} were examined using a variation of the technique of differential solubility thermal gel analysis (Rigell & Freire, 1987). Briefly, this technique takes advantage of the existence of characteristic solubility changes in the various subunits of a multisubunit protein upon unfolding and allows one to separate them by centrifugation. Subsequent analysis is done by gel electrophoresis of the pellet and supernatant.

Figure IV-7 shows typical laser densitometer tracings of SDS-polyacrylamide gels of the supernatant of cholera toxin samples heated up to the indicated temperature and then centrifuged as indicted in the methods section. At low temperatures, the densitometer traces are characterized by the presence of two bands corresponding to the B subunit monomer and A subunit respectively. The A subunit peak is present in the free cholera toxin samples heated up to 65°C and absent

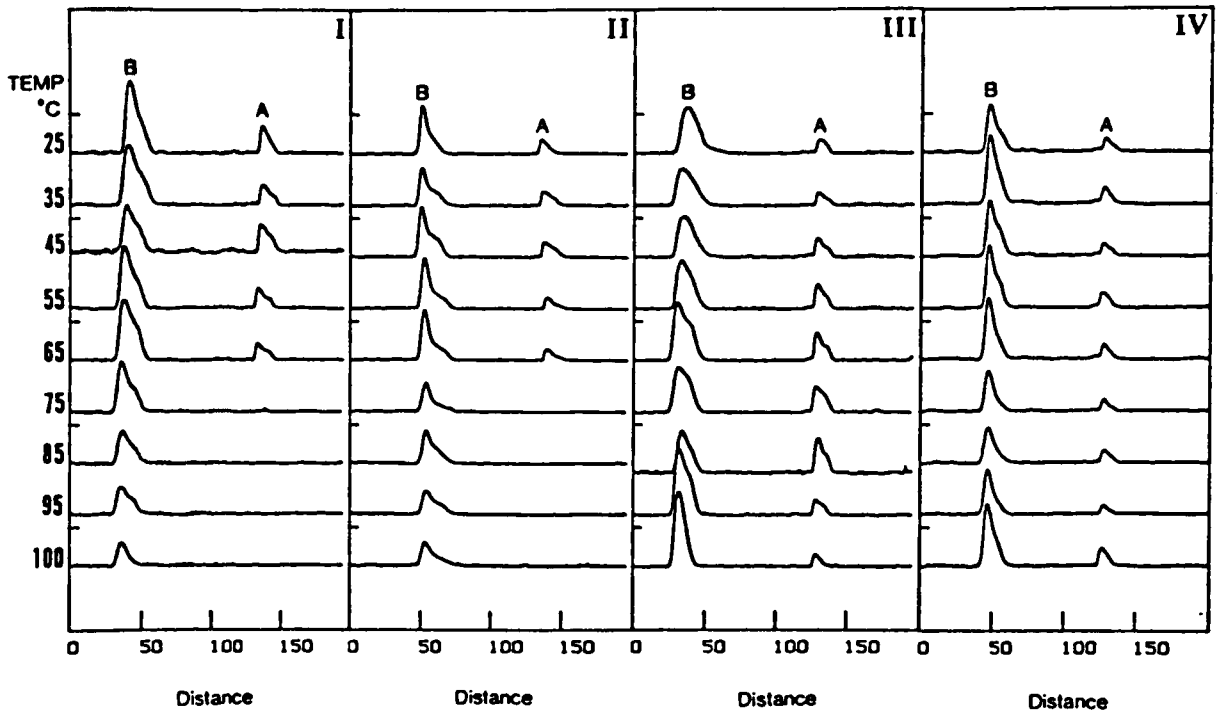

Figure IV-7. Laser densitometer tracing of differential solubility gel analysis experiment of cholera toxin in solution (panel I), in the presence of egg PC small sonicated vesicles (panel II), in the presence of ganglioside G_{M1} micelles (panel III) or in the presence of egg PC small sonicated vesicles containing 10 mol % of ganglioside G_{M1} (panel IV).

in samples heated to higher temperatures (Figure IV-7, panel I). The disappearance of the A subunit peak coincides with the presence of a white pellet in the samples heated above 65°C. Furthermore, this disappearance coincides with the exothermic effect seen in the calorimetric scans of cholera toxin in solution. These results indicate that the A subunit dissociates from the intact toxin molecule at the onset of B subunit pentamer denaturation. Following dissociation, precipitation of the A subunit occurs because the A subunit is a hydrophobic protein. By contrast, the B subunit remains soluble in aqueous solution even at temperatures above denaturation.

The dissociation and precipitation of the A subunit are also evident in the densitometer tracing of cholera toxin samples incubated with lipid vesicles lacking ganglioside G_{M1} (Figure IV-7, panel II). On the contrary, the A subunit remains in the supernatant in those samples incubated with ganglioside G_{M1} either in micellar form (Figure IV-7, panel III) or incorporated into lipid bilayers (Figure IV-7, panel IV). Since the A subunit precipitates when free in aqueous solution, these results indicate that the A subunit stays associated to the ganglioside micelles or the phospholipid vesicles containing ganglioside G_{M1} but not to the phospholipid vesicles lacking ganglioside. The calorimetric results agree with this observation since no exotherm was seen in the scans when ganglioside G_{M1} was present.

Figure IV-8 presents the results of a quantitative analysis of the differential solubility experiments. In Figure IV-8 the peak areas corresponding to the A subunit were normalized with respect to

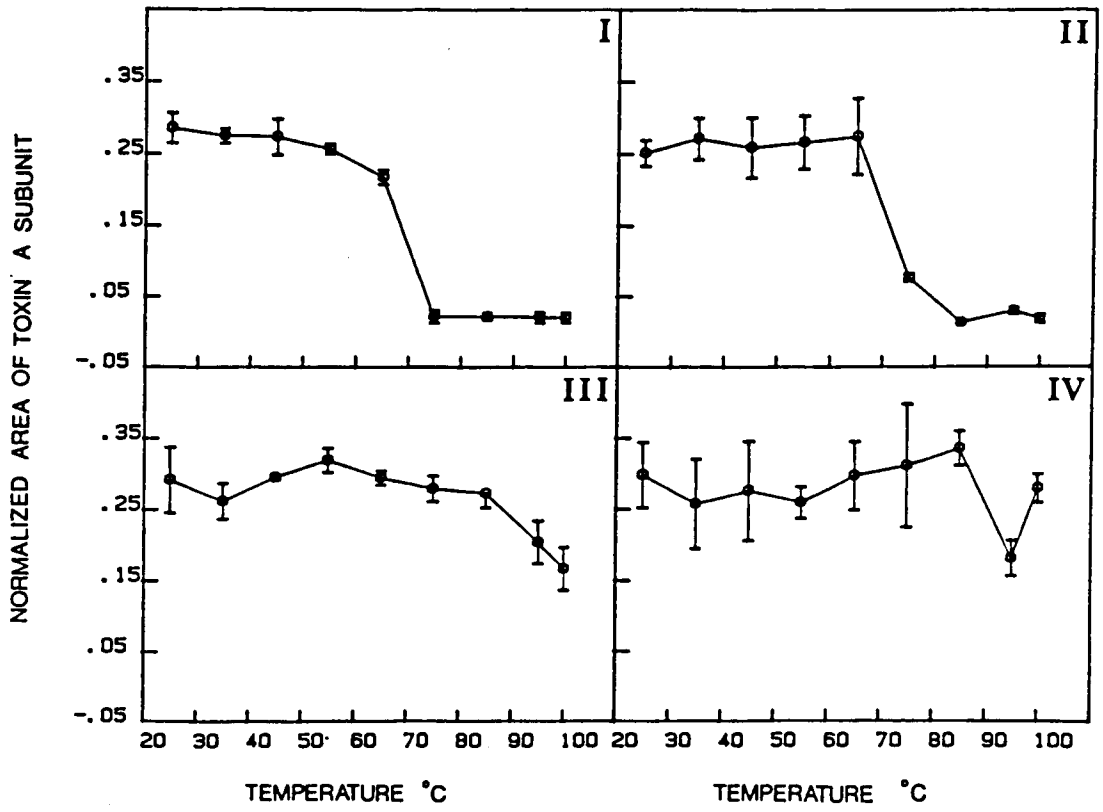

Figure IV-8. Normalized peak areas versus temperature calculated from differential solubility gel analysis experiments for cholera toxin alone (panel I), in the presence of lipid vesicles (panel II), in the presence of ganglioside G_{M1} micelles (panel III) or in the presence of lipid vesicles containing 10 mol % G_{M1}. Cholera toxin A subunit areas were normalized to account for any errors incurred from sample handling of dividing the respective area of the A subunit by the toxin B subunit area. The points are the average values obtained from six (I), three (III) or two (II, IV) separate experiments. The error bars represent the standard error of the mean at each temperature point.

the area of the B subunit and plotted as a function of temperature. As shown in Figure IV-8, dissociation and precipitation of the A subunit occur when the intact toxin is free in solution or in the presence of pure phospholipid vesicles, but not when incubated with micellar ganglioside G_{M1} or phospholipid vesicles containing ganglioside G_{M1}. The midpoint for the A subunit dissociation and precipitation occurs at 70°C, i.e. three degrees below the transition midpoint of the B subunit unfolding. The results indicates that the A subunit is released into solution at the onset of the thermal unfolding of the B subunit pentamer. In the presence of ganglioside G_{M1}, the A subunit stays associated to the micelles or lipid vesicles and does not precipitate out of solution after the unfolding of the B subunit. At this point, however, it is not clear whether the A subunit is free within the hydrophobic core of the micelle/bilayer or whether it remains attached to the B subunit. Additional gel filtration experiments (data not shown) indicate that the B subunit elutes together with the ganglioside even in samples that have been incubated at postdenaturational temperatures, suggesting that the B subunit also remains associated to the micelles or lipid vesicles.

High Sensitivity Isothermal Reaction Calorimetry

The energetics of the interaction between cholera toxin and ganglioside G_{M1} was measured directly using isothermal reaction calorimetry. This interaction can be divided into two steps: 1) binding of the B subunit to the oligosaccharide moiety of ganglioside G_{M1} and 2) penetration of the active A subunit into the hydrophobic

region of the membrane. These two processes can be measured using the intact toxin and the B subunit pentamer.

The results of a typical titration experiment are shown in Figure IV-9. Panel I shows the results of subsequent injections of intact cholera toxin into excess ganglioside G_{M1} micelles in order to ensure complete association of the toxin. Analysis of the data yields an enthalpy of association of -71.5 ± 5 Kcal/mole of toxin. Similar experiments were performed using isolated B subunit pentamer and the results are shown in Panel II. In this case the enthalpy of association was -49.4 ± 4 Kcal/mole of pentamer. It should be noted that these thermodynamic values take into account the sample heat of dilution produced during the injection. As shown in the bottom trace of panel I, the heat released following addition of the sample into buffer alone can be quantitated and subtracted from the overall heat produced during each toxin-ganglioside G_{M1} titration.

Three major points can be concluded from these data. First, the results of the toxin B pentamer experiment suggest that the binding subunit-ganglioside G_{M1} association only induces a small conformational change on the toxin molecule. The binding enthalpy of the B pentamer is approximately -10 Kcal/mole of ganglioside which is not much larger in magnitude than the expected value if the process were a simple protein-saccharide binding reaction. For example, Dani et al. (1981) determined an enthalpy change of -5.5 Kcal/mole for the association of concanavalin A to methyl-α-D-mannopyranoside. Second, the 20 Kcal/mole exothermic enthalpy difference between the enthalpy of association of intact toxin and B pentamer is most probably due to

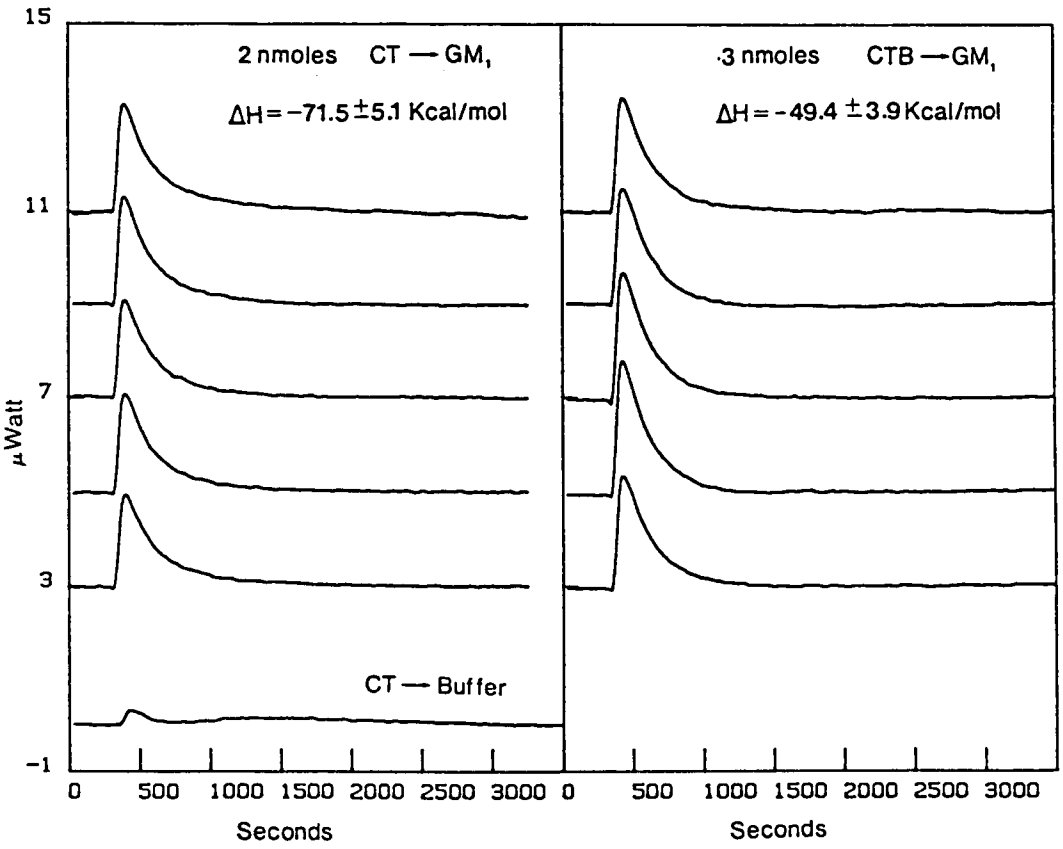

Figure IV-9. Power output (uwatts) as a function of time (seconds) for successive injections of intact cholera toxin (panel I) or isolated B pentamer (panel II) at equal intervals of 1 hour. Curves are offset for presentation purposes. The calorimetric cell contained excess ganglioside G_{M1} (1 umole in 1 ml) in order to ensure complete association of the toxin.

the penetration of the A subunit into the hydrophobic region of the micelle. Previous results of other protein-membrane systems studied in Dr. Freire's laboratory have shown large exothermic heat effects when a protein was transferred from an aqueous environment and inserted into the lipid bilayer (Ramsay et al., 1986; Myers et al., 1987). These heat effects have several origins namely conformational changes of the protein upon insertion, lipid hydrocarbon chain perturbation upon protein insertion or degree of hydration differences. Since the A subunit is already located within a hydrophobic environment within the B pentamer, lipid hydrocarbon chain perturbation and protein conformational differences should account for most of the energy of insertion. Last, we estimate a binding entropy of -200 cal/K·mol for the association of cholera toxin to ganglioside G_{M1} using our calorimetrically measured binding enthalpy and association constant in the range of 10^8 - 10^{10} M (Sattler et al., 1978; Gill, 1977). The sign and magnitude of the calculated entropy change indicates a large reduction in the molecular degrees of freedom which can not be accounted for by a positive contribution like the release of water from the oligosaccharide head group upon binding. This observation is consistent with the increase in cooperativity of unfolding observed upon ganglioside binding and suggests that the ganglioside-bound B pentamer is more rigid than the ganglioside-free B pentamer.

4. DISCUSSION

Cholera toxin is a multisubunit protein consisting of two

structurally distinct subunits, A and B. Protomer B is a pentameric ring of identical subunits which bind ganglioside G_{M1} with high affinity. Protomer A consists of two different subunits, A1 and A2, linked by a disulfide bond. The subunits are arranged such that the A subunit occupies the central channel of the B subunit pentamer extending well above the plane of the pentameric ring (Dwyer and Bloomfield, 1982; Ribi and Kornberg, 1987). The hydrophobic A subunit is the toxic component capable of activating adenylate cyclase. Since the A subunit does not bind cells, it must rely on the B component for association to ganglioside G_{M1} molecules on the cell membrane surface.

As distinct structural subunits, the A and B components of intact toxin are characterized by different thermotropic behaviors. This is evidenced by the existence of two well separated thermal transitions in the heat capacity profile. The A subunit unfolds at $51^{o}C$ with an enthalpy change of 90 Kcal/mole whereas the B subunit pentamer unfolds at $74^{o}C$ with an enthalpy change of 520 Kcal/mol of pentamer. The presence of the A subunit does not significantly affect the thermal unfolding of the B subunit pentamer, since within the experimental error, identical thermotropic behavior is found for the B subunit whether in isolated form or as a part of the holotoxin. Thus, the noncovalent interactions between the A component and the B subunit pentamer appear to be of a local nature. These results indicate that the A and B subunits behave independently of each other and that their thermodynamic stability is determined by intra rather than heterologous intersubunit interactions.

The calorimetric studies in conjunction with differential solubility thermal gel analysis have allowed us to follow the mechanism of thermal denaturation of intact cholera toxin. In the absence of ganglioside G_{M1}, this event consists of 1) unfolding of the A subunit while attached to the holotoxin; 2) dissociation of the A subunit at the onset of B subunit pentamer unfolding and 3) precipitation of the dissociated A subunit. The dissociation of the A subunit also supports the idea that the forces between the A and B components are derived from weak interactions. Most likely, the intact B subunit pentamer provides a hydrophobic cavity where the A subunit partitions. When this cavity is destroyed at the onset of the B pentamer unfolding the A subunit is released and precipitates out of solution. Moreover, these results also indicate that a major function of the B subunit pentamer is to act as a solubilization agent facilitating the movement of the A subunit through an aqueous environment.

The thermotropic behavior of cholera toxin is greatly influenced by ganglioside G_{M1} as shown by a dramatic increase in the transition temperature of the B subunit pentamer. This same behavior has been reported (Dalziel et al., 1984) for the isolated B subunit pentamer suggesting that the behavior of the B subunit is not affected by the presence of the A subunit. By contrast, incubation of intact toxin with ganglioside G_{M1} results in only a slight increase in the transition temperature for the A subunit transition. The results from several spectroscopic investigations (Fishman et al., 1978; Sillerud et al., 1981; Tomasi et al., 1984) have suggested the existence of a

conformational change in the B subunit upon binding to ganglioside G_{M1}. The calorimetric results presented in this section revealed that the major effect of ganglioside is on the cooperative interactions between the B subunits and demonstrate further that there is little, if any, direct interaction between the A subunit and ganglioside G_{M1}. This conformational difference probably does not represent a gross structural rearrangement (Dwyer & Bloomfield, 1982) but rather local amino acid side chain reorientation due to ganglioside G_{M1} association (Tomasi et al., 1984). The isothermal titration calorimetry results also suggest that ganglioside G_{M1} only induces a small conformational change on the toxin.

The association of ganglioside G_{M1} to cholera toxin enhances the cooperative interactions within the B subunit pentamer such that fully bound pentamer unfolds as a single entity. Similar cooperative behavior occurs with isolated B subunit pentamer indicating that the A subunit does not mediate the intersubunit interactions within the B pentamer. The binding site for the ganglioside G_{M1} has been postulated as being at the interface between B subunit monomers (DeWolf et al., 1981). These results suggest that there is communication between two adjacent B monomers and that this additional intersubunit communication in the presence of ganglioside G_{M1} may play a role in the insertion of the A subunit into the membrane.

The A subunit unfolding transition is irreversible whereas the B subunit pentamer transition is reversible. Although previously believed to undergo an irreversible thermal denaturation (Dalziel et

al., 1984), the reversibility of the B subunit pentamer is dependent on the amount of time the sample is incubated at post-transitional temperatures.

Intact toxin A subunit does not precipitate at the onset of the B subunit unfolding in the presence of ganglioside G_{M1} suggesting a hydrophobic interaction between the A subunit with the lipophilic core of ganglioside G_{M1}. This interaction is initiated by the binding of the B subunit pentamer to the oligosaccharide region of the ganglioside molecule, since the A subunit by itself is unable to bind ganglioside G_{M1}. This observation is consistent with the idea that the A subunit of cholera toxin when bound to ganglioside G_{M1} via the B component can insert directly into the lipid milieu. The postulated orientation of the toxin molecule upon association is with the A1 portion facing toward the membrane (Dwyer & Bloomfield, 1982). Previously, we have demonstrated (Goins & Freire, 1985) that the hydrophobic A1 subunit behaves as an integral membrane protein when incorporated into liposomes using standard membrane reconstitution techniques. Evidence for direct penetration has accumulated in the last few years using several techniques including hydrophobic photoaffinity labeling (Wisnieski & Bramhall, 1981; Tomasi & Montecucco, 1981; Tomasi et al., 1982), laser light scattering (Dwyer & Bloomfield, 1982), and lipid-layer crystallization (Ludwig et al., 1986; Ribi & Kornberg, 1987). Isothermal titration calorimetry results indicate that the insertion of the active A subunit into the interior of the membrane is an energetically favorable process. Within this context, the main role of the B subunit pentamer is to

provide a water soluble carrier to the A subunit and a mechanism to place it in close contact with the membrane. The mechanism involves ganglioside G_{M1} as a high affinity membrane surface receptor and as a modulator of the intersubunit interactions within the cholera toxin molecule.

PART IV

REFERENCES

REFERENCES

Dalziel, A. W., Lipka, G., Chowdhry, B. Z., Sturtevant, J. W., and Schafer, D. E. (1984) Mol. Cell. Biochem. 63: 83-91.

Dani, M., Manca, F. and Rialdi, G. (1981) Biochim, Biophys. Acta 667: 108-117.

DeWolf, M. J. S., Fridkin, M., and Kohn, L. D. (1981) J. Biol. Chem. 256: 5489-5496.

Dwyer, J. D., and Bloomfield, V. A. (1982) Biochemistry 21: 3231-3234.

Felgner, P. L., Freire, E., Barenholz, Y., and Thompson, T. E. (1981) Biochemistry 20: 2168-2171.

Fishman, P. H., Moss, J., and Osborne, J. C., Jr. (1978) Biochemistry 17: 711-716.

Gill, D. M. (1976) Biochemistry 15: 1242-1248.

Gill, D. M. (1977) Adv. Cyclic Nucleotide Res. 8: 85-118.

Goins, B., and Freire, E. (1985) Biochemistry 24: 1791-1797.

Kates, M. (1972) Techniques of Lipidology. pp. 502-579, North-Holland Publishing Co., Amsterdam.

Laemmli, U. K. (1970) Nature (London) 227: 680-685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193: 265-275.

Ludwig, D. S., Ribi, H. O., Schoolnik, G. K., and Kornberg, R. D. (1986) Proc. Natl. Acad. Sci. U.S.A. 83: 8585-8588.

Masserini, M., and Freire, E. (1987) Biochemistry 26: 237-242.

Mullin, B. R., Aloj, S. M., Fishman, P. H., Lee, G. Kohn, L. D., and Brady, R. O. (1976) Proc. Natl. Acad. Sci. U.S.A. 73: 1679-1683.

Myers, M., Wortman, C., and Freire, E. (1984) Biochemistry 23: 1442-1448.

Myers, M., Mayorga, O. L., Emtage, J. and Freire, E. (1987) Biochemistry 26: 4309-4315.

Ramsay, G., Prabhu, R. and Freire, E. (1986) Biochemistry 25: 2265-2270.

Ribi, H. O., and Kornberg, R. D. (1987) Biophys. J. 51: 168a.

Rigell, C. W., and Freire, E. (1987) Biochemistry. 26: 4366-4371.

Sattler, J., Schwarzmann, G., Knack, I., Rohm, K. H. and Wiegandt, H. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359: 719-723.

Schullery, S. E., Schmidt, C. F., Felgner, P., Tillack, T. W., and Thompson, T. E. (1980) Biochemistry 19: 3919-3923.

Sillerud, L. O., Prestegard, J. H., Yu, R. K., Konigsberg, W. H., and Schafer, D. E. (1981) J. Biol. Chem. 256: 1094-1097.

Spiro, R. G. (1966) Methods Enzymol. 8: 1-52.

Thompson, T. E., Johnson, M., Allietta, M., Brown, R. E., and Tillack, T. W. (1985) Biochim. Biophys. Acta 817: 229-237.

Tomasi, M., and Montecucco, C. (1981) J. Biol. Chem. 256: 11177-11181.

Tomasi, M., Battistini, A., Cardelli, M., Sonnino, S., and D'Agnolo, G. (1984) Biochemistry 23: 2520-2526.

Tomasi, M., D'Agnolo, G., and Montecucco, C. (1982) Biochim. Biophys. Acta 692: 339-344.

Van Heyningen, W. E., and Seal, J. R. (1983) Cholera: The American Scientific Experience 1947-1980. Westview Press, Boulder, CO.

Wisnieski, B. J., and Bramhall, J. S. (1981) Nature (London) 289: 319-321.

CONCLUSION

CONCLUSION

This dissertation has dealt with the precise quantitative thermodynamic characterization of the sequence of events comprising the association and insertion of cholera toxin into membranes. The steps involved are 1) association of toxin to ganglioside G_{M1}, the toxin cell surface receptor and 2) release and penetration of the toxin bioactive (A) subunit into receptor-containing membranes.

The effect of ganglioside G_{M1} association on toxin structure and function was investigated. High sensitivity differential scanning calorimetry (DSC) experiments indicated that ganglioside G_{M1} association stabilized the toxin molecule by shifting the binding (B) subunit transition temperature upwards twenty degrees and by modulating the cooperative intersubunit interactions of the B subunit. It is plausible that this ganglioside induced enhancement of the cooperative interactions of the B subunit pentamer may play a role in the toxin insertional mechanism by triggering the release of the A subunit. The A subunit would then be placed in closer contact with the membrane and free to partition into the hydrophobic core of the lipid bilayer. Differential solubility thermal gel analysis experiments showed a hydrophobic interaction between the A subunit and the lipophilic matrix of ganglioside micelles or bilayers containing ganglioside G_{M1}, which is in agreement with the toxin thermal denaturation studies.

The association and insertion of cholera toxin into phospholipid-G_{M1} bilayers was also characterized by combining DSC with

other fluorescent techniques. The results of these studies showed that the initial association of intact cholera toxin had little effect on the parameters used to monitor membrane physical state. This study also allowed us to define the topology of the individual subunits during the insertional event. The B subunit associated to and remained absorbed to the membrane surface whereas the hydrophobic A subunit penetrated the membrane directly, perturbing the hydrophobic core region of the membrane. In addition, this insertional event was facilitated under conditions of lateral phase separation induced by calcium, demonstrating the importance of lipid lateral organization in triggering a particular event such as the insertion of a protein into a membrane.

In conclusion, we have used the interaction of cholera toxin with its cell surface receptor, ganglioside G_{M1} as a model system to study the association and insertion of a protein into membranes. This study characterized for the first time the interactions of a ligand-receptor pair at a detailed molecular level. It is our hope that this study will set a precedent for future thermodynamic characterizations of the interactions between proteins and lipids.

VITA

Beth Ann Goins was born in Sullivan County, Tennessee on February 20, 1959. She is a 1976 graduate of Ketron High School located in Kingsport, Tennessee. In May of 1980 Beth received her B.S. in Chemistry from King College, Bristol, Tennessee. Following her graduation from King, she entered the graduate program in Biology at East Tennessee State University where she continued her studies for two years.

In September of 1982 Beth enrolled in the graduate program in Biochemistry at the University of Tennessee, Knoxville. She received an M.S. in Biochemistry in August 1985 followed by a Ph.D. in the same subject in June 1988.