Subunit Arrangement of Cholera Toxin in Solution and Bound to Receptor-Containing Model Membranes†

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ABSTRACT: Quasi-elastic laser light scattering (QLS) is used to study the translational frictional properties of cholera toxin and its complex with ganglioside G_{m1} receptor containing phospholipid vesicles. These properties are compared to theoretically calculated values for model structures composed of spherical subunits in order to assess the structural configuration of the toxin and its binding geometry on membrane surfaces. The structure for the toxin that best fits the experimental results consists of the five B subunits arranged radially about an elongated A subunit, which extends well above the plane of the B subunits. Binding of cholera toxin to G_{m1}-containing model membranes results in a complex in which the B subunits are absorbed on the surface while the A subunit penetrates the membrane bilayer.

Over the past 10 years the protein subunit composition of cholera toxin has been well established (Finkelstein et al., 1972; van Heyningen, 1976; Lai, 1980). It consists of six subunits: five B subunits, each capable of binding a membrane receptor, and one A subunit, which possesses the enzymatic activity of the toxin. The A subunit is in turn composed of two polypeptide chains, the A_1 and A_2 fragments. Although it has generally been accepted that the cholera toxin molecule possesses 5-fold symmetry about an axis perpendicular to the plane of the B pentamer (Gill, 1977; Sigler et al., 1977; Lai, 1980), a quantitative description of the dimensions of the molecule as well as the spatial arrangement of the A subunit with respect to the B subunits has been lacking. Furthermore, it is not established whether, during the initial process of binding to receptor-containing membranes, cholera toxin undergoes any major subunit rearrangements or penetrates the bilayer in any way. Gill (1977) has suggested that subsequent to binding both the A and B subunits enter the membrane although this has recently been disputed by Wisnieski & Bramhall (1981), who found that only the A subunit entered the bilayer.

An effective method for assessing macromolecular structures and dimensions in solution is to compare calculated hydrodynamic fractional properties of an assumed model structure with experimentally determined ones. Accurate results for realistic models of globular proteins are obtained when the structure is modeled by a collection of spherical subunits of variable size (Teller et al., 1980). Hydrodynamic frictional properties of such structures are readily calculated by using well-developed hydrodynamic theory (Garcia de la Torre & Bloomfield, 1977, 1981; Garcia de la Torre & Garcia Bernal, 1980). Adjustments in the number, size, and position of the frictional subunits may be made to obtain the best agreement with experiment.

A technique that had proved to be effective in experimentally determining hydrodynamic frictional properties is quasi-elastic laser light scattering (QLS).[1] In the simplest application of this method, the time autocorrelation function of the photocurrent produced by the scattered light is analyzed to obtain the translational diffusion coefficient (Berne & Pecora, 1976; Bloomfield & Lim, 1978).

In this paper we utilize these experimental and theoretical hydrodynamic techniques to characterize cholera toxin both free in solution and bound to its receptor, the ganglioside G_{m1} (van Heyningen et al., 1971; Cuatrecasas, 1973), incorporated in spherical phospholipid vesicles. The structural model we obtain for cholera toxin is used in the interpretation of hydrodynamic data for cholera toxin–vesicle complexes in an effort to probe for structural rearrangements or membrane penetration during the initial binding state of the interaction. A similar approach has been used previously to study prothrombin and its membrane-bound complex (Lim et al., 1977).

We begin with a model for cholera toxin similar to that proposed previously (Gill, 1977; Lai, 1980) in which the five B subunits are arranged radially about a centrally located A subunit. Adjustment in the mass distribution of the A subunit on a central axis normal to the plane of the B subunits is made until the calculated translational frictional coefficient most closely matches the experimental value. We show below that this occurs when the A subunit extends well above the B plane. This model possesses an inherent sidedness with respect to membrane binding, leading to two possible orientations on the membrane surface. Our results for membrane-bound cholera toxin indicate that the toxin binds to membrane surfaces with the B subunits remaining absorbed to the surface, while the A subunit is incorporated into the membrane. Chemical cross-linking studies indicate that incorporation of the A subunit into the membrane is not the result of any major subunit rearrangements of the protein on the membrane surface. It is interpreted instead as the result of a hydrophobic interaction between the A_1 fragment and the hydrocarbon core of the bilayer.

Materials and Methods

Cholera Toxin. Cholera toxin was obtained from Schwarz/Mann as a lyophilized powder. When rehydrated

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[1] Abbreviations: QLS, quasi-elastic laser light scattering; G_{m1}, Galβ1→3GalNAcβ1→4Gal(3←2αAcNeu)β1→4Glcβ1→1′Cer; DTBP, dimethyl 3,3′-dithiobis(propionimidate); PC, phosphatidylcholine; TLC, thin-layer chromatography; $NaDodSO_4$, sodium dodecyl sulfate; $A_2$5B, cholera toxin without A_1 fragment; CT, cholera toxin; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

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Table I: Subunit Structure of Cholera Toxin

subunit	M_r	radius (Å)
B	11500	16.7
A_2	5000	12.7
A_1 (as monomer)	21500	20.6
A_1 (as dimer)	2 × 10750	16.4

in buffer (0.1 M NaCl, 0.05 M Tris, pH 7.0), the toxin was found to be in a highly aggregated state even at a concentration of 1 mg/mL. Monodisperse toxin was obtained by diluting the toxin to 10 μg/mL, followed by incubation for at least 3 days at 4 °C. Reconcentration (to a concentration of roughly 1 mg/mL) was performed by using a Millipore CX-10 ultrafilter system attached to a vacuum pump. Following this procedure the toxin was greater than 90% monomeric as judged by light scattering molecular weight determination and showed little tendency to reaggregate.

Preparation of the $A_2$5B complex was achieved by treating cholera toxin with a 5-fold molar excess of mercaptoethanol and incubating at 37 °C overnight (Gill, 1977). The reductively cleaved A_1 fragment formed a white precipitate that was separated by centrifugation at 10000 rpm for 2 h. The A_1 pellet was resuspended in buffer and repelleted to remove any last traces of $A_2$5B. This procedure worked equally well with aggregated and unaggregated cholera toxin, implying that the aggregation was through the A_1 fragment.

Intramolecular cross-linking of the subunits of cholera toxin was performed by using dimethyl 3,3′-dithiobis(propionimidate) (DTBP) as the cross-linking reagent (Davies & Stark, 1970). A 300-fold molar excess of DTBP was added to cholera toxin (250 mg) in 1 mL of 0.2 M triethanolamine buffer, pH 8.5. The reaction was allowed to proceed at room temperature for 45 min at which time it was quenched with 0.25 M ammonium acetate.

Ganglioside G_{m1}. Mixed beef brain gangliosides were obtained by the method of Svennerholm (1972) and converted to monosialogangliosides with a neuraminidase digest as described by Sillerud et al. (1978). About 150 mg of the crude G_{m1} so obtained was applied to a 1.5 × 25 cm column of DEAE-Sepharose (acetate form) in CH_3Cl–CH_3OH–H_2O (30:60:8). After elution with 1 column volume each of this solvent and of methanol, G_{m1} was eluted with 2.5 column volumes of 0.07 M ammonium acetate in methanol. The solvent in this last fractionation was evaporated off and the product dissolved in 25 mL of water and dialyzed against distilled water. Following lyophilization, TLC showed G_{m1} to be the only species present.

Preparation of Phosphatidylcholine–G_{m1} Vesicles. Phosphatidylcholine (PC) was obtained from Sigma and used without further purification. Monodisperse PC–G_{m1} bilayer vesicles were prepared by the ethanolic injection method of Kremer et al. (1977). G_{m1} (80 μg) in CH_3Cl–CH_3OH (1:1) and PC (2 mg) in hexane were mixed, and the solvent was removed. The residue was dissolved in 0.5 mL of absolute ethanol. Of this, 0.4 mL was injected with stirring into 8.5 mL of buffer at 40 °C. Prior to use, the vesicles were dialyzed overnight to remove the alcohol.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis was performed on 15% polyacrylamide gels in a slab-gel apparatus as described by Laemmli (1970). Samples were run under nonreducing conditions.

QLS Measurements. The instrumentation and data analysis methods used in the QLS measurements have been described elsewhere (Pletcher et al., 1980). Sample volumes of 0.75 mL were used, and dust was removed by centrifuging at 8000 rpm for 40 min in a Beckman J-21B centrifuge.

Hydrodynamic Calculations. Theoretical values for translational frictional coefficients were obtained by the Gauss–Seidel iterative method as described by Garcia de la Torre & Bloomfield (1977, 1981) and Garcia de la Torre & Garcia Bernal (1980). The reader is directed to these references for a treatment of the theoretical formalism.

Results

Subunit Arrangement of Cholera Toxin. Table I is a summary of the subunit structure used in modeling cholera toxin. The molecular weights of the subunits have been previously determined (Gill, 1977), and their hydrated radii were calculated from the molecular weights by using the equation

$$R = [[3M_r/(4\pi N_a)](\bar{v}_2 + \delta_1\bar{v}_1^0)]^{1/3}$$

where M_r is the molecular weight, N_a is Avogadro's number, $\bar{v}_2$ is the subunit partial specific volume, δ_1 (grams of H_2O per gram of protein) is the hydration of the subunit, and $\bar{v}_1^0$ is the partial specific volume of water. In these calculations $\bar{v}_2$ = 0.73 mL/g, $\bar{v}_1^0$ = 1.0 mL/g, and δ_1 = 0.3 g/g, typical values for globular proteins.

The basic structural model we employ for cholera toxin has been previously suggested (Gill, 1977; Lai, 1980). It consists of the five B subunits radially arranged about an axially located A subunit. In particular, we assume all A–B contacts to be through the A_2 fragment with the A_1 fragment then projecting out one side of the molecule. In subsequent calculations the A_1 fragment is considered to be either a single spherical subunit or a more elongated structure represented by two smaller spherical subunits. These two model structures are shown in Figure 1 along with their overall dimensions.

Table II lists calculated and experimental translational hydrodynamic properties for both the $A_2$5B complex and cholera toxin. For cholera toxin, theoretical values for both of the structures shown in Figure 1 are presented. Included here are the translational diffusion coefficient $D_{20,w}$ and the ratio f/f_0:

$$f/f_0 = R_h[3M_r(\bar{v}_2 + \delta_1\bar{v}_1^0)/(4\pi N_a)]^{-1/3}$$

where the hydrodynamic radius is given by $R_h = kT/(6\pi\eta_0 D_{20,w})$. The ratio f/f_0 reflects the asymmetry of the molecule and is the ratio of the actual frictional coefficient to that of a spherical molecule of equal volume.

The excellent agreement between the calculated and experimental hydrodynamic properties for the $A_2$5B complex strongly supports our model for this structure. Although

Table II: Translational Hydrodynamic Properties of $A_2$5B and Cholera Toxin

	calcd			exptl	
	$A_2$5B	CT (A_1 as one sphere)	CT (A_1 as two spheres)	$A_2$5B	CT
$D_{20,w}$	6.71 × 10^{-7}	5.95 × 10^{-7}	5.64 × 10^{-7}	6.79 × 10^{-7}	5.76 × 10^{-7}
f/f_0	1.09	1.11	1.17	1.07	1.15

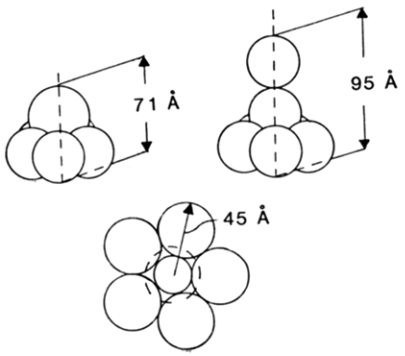

FIGURE 1: Models for subunit configuration of cholera toxin. The A_1 fragment is represented by a single spherical element (top left) or by two slightly smaller spherical elements (top right). The bottom drawing shows a view along the 5-fold axis from below.

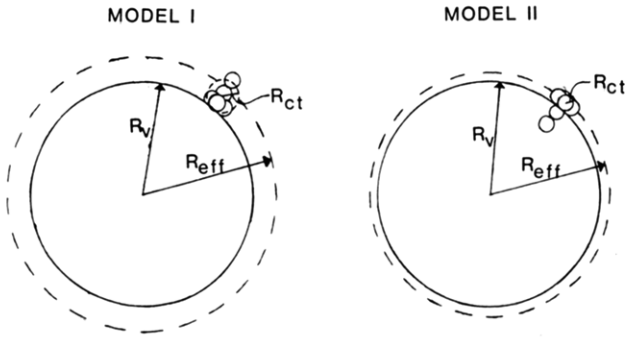

FIGURE 2: Models for the two possible membrane binding geometries of cholera toxin. R_v is the vesicle radius, R_{ct} is the equivalent radius of the bound cholera toxin, and R_{eff} is the effective radius of the cholera toxin–vesicle complex. R_{ct} values for models I and II are 37.3 and 16.7 Å, respectively.

neither model structure predicts the translational frictional properties of the whole toxin, the model with the more elongated A_1 fragment is in slightly closer agreement and is within the 2% uncertainty in the diffusion coefficient measurements.

Cholera Toxin Bound to G_{m1}-Containing Vesicles. Inherent in our proposed model of cholera toxin is a sidedness with respect to membrane binding. As all of the B subunits are assumed to bind G_{m1} receptors, the toxin may bind with the A_1 fragment directed either out into solution or down into the membrane. These two possibilities are shown in Figure 2. The vesicle is represented by a large sphere of radius R_v, and the bound toxin molecules are represented as equivalent spherical frictional elements of radius R_{ct}. In model I, where the A_1 fragment is directed out into solution, R_{ct} will be very close to the hydrodynamic radius of the free toxin, 37.3 Å, since the toxin is relatively symmetric. In model II, R_{ct} is given by the radius of a B subunit, 16.7 Å. For these types of binding geometry, hydrodynamic theory (Bloomfield et al., 1967; McCammon et al., 1975) predicts that the effective radius, R_{eff}, of the vesicle–cholera toxin complex will be given by

$$R_{eff} = [R_v + (f/f_p)R_{ct}][1 + 0.25(f/f_p)R_{ct}/[R_v + (f/f_p)R_{ct}]] \quad (1)$$

where f/f_p is an empirical function of the fraction of the vesicle surface covered by toxin. Once more than half of the surface is covered, f/f_p is essentially unity. This was the case in our studies in the plateau region, where coverage was typically 65%.

The experimentally observed change in vesicle radius as a function of added protein is shown in Figure 3 for both the $A_2$5B and cholera toxin–vesicle complexes. Also shown are theoretical curves for the two possible binding geometries of

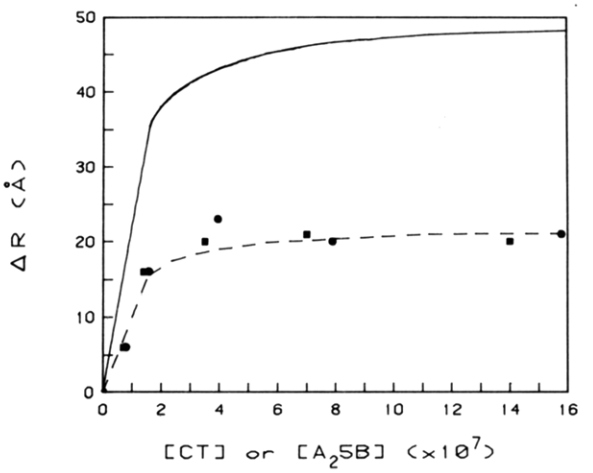

FIGURE 3: Change in PC–G_{m1} vesicle radius as a function of added $A_2$5B (●) and cholera toxin (■). Lines represent theoretical predictions of eq 1 for the binding geometry of model I (—) and model II (– –).

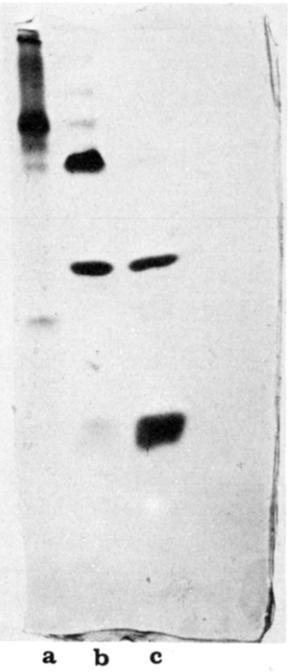

FIGURE 4: NaDodSO$_4$–polyacrylamide gel electrophoresis of (a) DTBP cross-linked cholera toxin, (b) unboiled cholera toxin, and (c) boiled cholera toxin. Each sample represents about 10 mg of protein.

Figure 2 as predicted by eq 1. Both the $A_2$5B and the whole toxin complexes exhibit virtually identical hydrodynamic behavior, and hence also binding geometries, and the diffusion coefficients are well described by the model in which the A_1 fragment is incorporated into the membrane bilayer (model II).

Although it appears that the A_1 fragment is inserted into the membrane when cholera toxin binds to receptor-containing vesicles, the above experiments do not illuminate the mechanism of this process. It is conceivable that the toxin could initially bind as in model I and subsequently undergo a subunit rearrangement, allowing the A subunit to pass through or around the B subunits, thus entering the membrane. So that distinction could be made between this mechanism and one in which the A_1 penetration is coincident with receptor binding, the subunits of cholera toxin were covalently cross-linked with DTBP prior to membrane binding. Such cross-linking should eliminate any subunit rearrangements thus locking the bound toxin in its initially bound state.

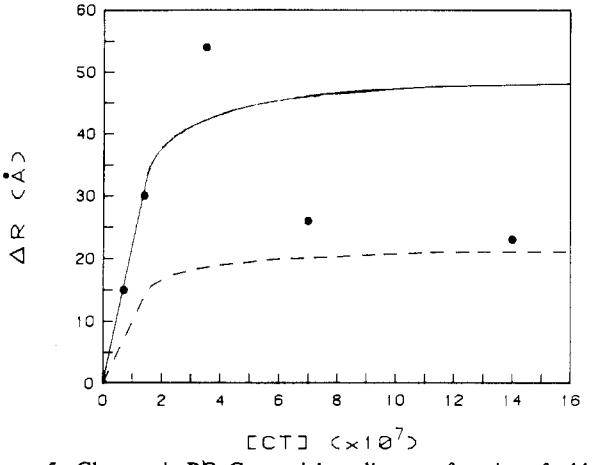

FIGURE 5: Changes in PC–G_{m1} vesicle radius as a function of added cross-linked cholera toxin (●). Lines represent theoretical predictions of eq 1 for the binding geometry of model I (—) and model II (– –).

$NaDodSO_4$–polyacrylamide gel electrophoresis of the cross-linked cholera toxin is shown in Figure 4. Although some of the subunits remained un-cross-linked, the majority of the material was found in a 75000–85000 molecular weight band, consistent with totally cross-linked cholera toxin monomers. QLS of the cross-linked cholera toxin gave a hydrodynamic radius 5 Å larger than that of the native toxin, indicating a trace amount of intermolecularly cross-linked product.

The effect of binding cross-linked cholera toxin on the vesicle radius is shown in Figure 5. The difference between the shape of this isotherm and those in Figure 3 is attributable to agglutination of vesicles by the small amount of intermolecular cross-linked cholera toxin present. Nearly identical results were obtained when these vesicles were treated with noncovalently aggregated cholera toxin. This agglutination is similar to antibody–antigen precipitation in that it occurs only at intermediate protein concentrations. To agglutinate two vesicles requires a partially bound cholera toxin molecule or aggregate on one vesicle and a free receptor on the other. At low toxin concentrations the number of bound toxin molecules or aggregates is low, and thus the probability for the formation of a large number of intervesicle cross-links is low. At high concentrations, the vast majority of receptors will be occupied, and thus again the number of intervesicle cross-links will be low. Titration between these two regimes reveals a toxin concentration at which maximum agglutination occurs. At higher concentrations, agglutination is abolished as evidenced by an initial decrease and then leveling off of the change in vesicle radius attributable to bound toxin. This plateau region represents unagglutinated vesicles to which toxin is bound. The diffusion coefficient, and thus the orientation of the bound toxin on the vesicle surface in this plateau region, agrees very well with that predicted by model II. This indicates that no major subunit rearrangements take place on the membrane surface, so that the A_1 fragment must be inserted into the membrane when the B subunits bind to G_{m1} receptors. This strongly supports the idea that cholera toxin binds to membranes with the A_1 fragment directed toward the membrane surface.

Discussion

The purpose of these experiments was to apply modern hydrodynamic methodologies to the determination of the subunit configuration of cholera toxin, both free in solution and bound to a membrane surface. We hoped that this might provide insight into possible mechanisms whereby the A subunit is dissociated and passed across the membrane bilayer. Gill (1977) has suggested that the B subunits might participate in this process, subsequent to receptor binding, by penetrating the bilayer and creating a hydrophilic pore through which the A subunit may pass. This mechanism, however, is not consistent with our finding that the B subunits remain absorbed on the surface after the A subunit has penetrated the membrane. Results consistent with ours have been reported by Wisnieski & Bramhall (1981). Our experiments with cross-linked cholera toxin indicate that no major subunit rearrangements take place upon binding and that the A subunit penetrates the bilayer coincident with receptor binding. This is a result of the toxin's binding orientation, in which the A subunit is directed toward the membrane.

Perhaps a more likely role for the B subunits, in addition to receptor binding, is the solubilization of the A subunit in the aqueous environment. The hydrophobic nature of the A_1 fragment may be evidenced by its precipitation when reductively cleaved from the toxin. We have shown that when the B subunits bind to membrane receptors, they serve to localize the A_1 fragment very near the membrane surface. Furthermore, binding results in a cluster of five G_{m1} receptors. Such ordering of membrane components could very well alter the local lipid packing density, leading to disorder or disruption of the local membrane structure in which the hydrocarbon core of the membrane is exposed. This disruption could account for the increased membrane permeability to small molecules that results from cholera toxin or choleragenoid (B pentamer) binding (Moss et al., 1976, 1977). Incorporation of the A subunit into the membrane may then be driven by a hydrophobic interaction between the A subunit and the exposed hydrocarbon core of the bilayer. Conformational changes in the B subunits accompanying G_{m1} binding (Fishman et al., 1978) may serve to facilitate this process by reducing the strength of the A–B intersubunit interactions.

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Cholera Toxin Mediated Agglutination of Ganglioside G_{m1} Containing Phospholipid Vesicles and G_{m1}-Coated Polystyrene Spheres†

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ABSTRACT: Quasi-elastic laser light scattering is used to examine the cholera toxin mediated agglutination of ganglioside G_{m1} containing phospholipid vesicles and G_{m1}-coated polystyrene spheres. We find that the ability of cholera toxin to agglutinate G_{m1}-containing phospholipid vesicles depends markedly on the lipid composition of the vesicle, with only those composed of short-chain lipids (C14, C16) being appreciably agglutinated. This is interpreted as due to poor mixing of these lipids with the longer chain gangliosides, resulting in lateral separation of the gangliosides in the membrane bilayer. A simple quantitative model, a modification of that developed by von Schulthess et al. [von Schulthess, G. K., Cohen, R. J., Sakato, N., & Benedek, G. B. (1976a) *Immunochemistry 13*, 955–962], is developed to describe these agglutination processes. Application of this model to the agglutination of G_{m1}-coated polystyrene spheres by cholera toxin allows an estimate of a minimum value of 4.5×10^4 M^{-1} for the association constant of cholera toxin for its initial G_{m1} receptor.

Cholera toxin is a multimeric protein consisting of two types of subunits: an A subunit, which contains the enzymatic activity of the toxin, and five identical B subunits, each capable of binding a membrane receptor. The receptor for cholera toxin has been shown to be the ganglioside G_{m1}[1] (van Heyningen et al., 1971; Cuatrecasas, 1973). The binding of the toxin to cells and to liposomes containing G_{m1} has been heavily studied and is well reviewed (Bennett & Cuatrecasas, 1976; Gill, 1977; Lai, 1980).

In addition to binding receptors on a single cell, Richards et al. (1979) have demonstrated that the pentavalent nature of the molecule confers a lectin-like quality on cholera toxin. They were able to show that cholera toxin was capable of agglutinating erythrocytes and phospholipid vesicles containing G_{m1}. This agglutination resembled antibody–antigen precipitation, showing a maximum at intermediate cholera toxin concentrations.

A simplified thermodynamic model of such agglutination reactions has been formulated by von Schulthess et al. (1976a) and is the basis of their laser light scattering immunoassay (von Schulthess et al., 1976a,b, 1980). In this technique, based on the earlier work of Singer (1961), polystyrene (PS) latex spheres coated with antibody are agglutinated with divalent antigen. The degree of agglutination is sensitively monitored by changes in either the translational diffusion coefficient obtained from quasi-elastic laser light scattering (QLS) (von Schulthess et al., 1976a,b) or angular anisotropy as measured by total intensity light scattering (von Schulthess et al., 1980). In addition to the sensitivity of immunoassay, it is possible from the analysis of these data to extract association constants for the initial attachment of antibody to antigen as well as those for the cross-linking of two PS spheres.

In this paper we utilize modifications of the above technique to study the cholera toxin mediated agglutination of G_{m1}-containing phospholipid vesicles as well as G_{m1}-coated PS spheres. The major modifications consist of substituting receptor-containing phospholipid vesicles for receptor-coated PS spheres and allowing the pentavalent cholera toxin to bind more than one receptor per vesicle (or PS sphere). We find that the ability of cholera toxin to agglutinate G_{m1}-containing vesicles depends markedly on the lipid composition of the vesicle, with only those composed of short-chain lipids (C14, C16) being appreciably agglutinated. This is interpreted as due to poor mixing of these lipids with the longer chain gangliosides, resulting in lateral separation of the gangliosides in the membrane bilayer. The agglutination of G_{m1}-coated PS spheres by cholera toxin was analyzed to obtain an estimate of at least 4.5×10^4 M^{-1} for the association constant of cholera

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[1] Abbreviations: QLS, quasi-elastic laser light scattering; G_{m1}, Galβ1→3GalNAcβ1→4Gal(3←2αAcNeu)β1→4Glcβ1→1′Cer; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; DOPL, dioleoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; PS, polystyrene; G_{m1}-OS, oligosaccharide portion of G_{m1}; T_m, liquid-crystal to gel transition temperature; Tris, tris(hydroxymethyl)aminomethane.

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