Interaction of Cholera Toxin with Gangliosides: Differential Effects of the Oligosaccharide of Ganglioside G_{M1} and of Micellar Gangliosides†

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ABSTRACT: Ultraviolet difference absorption spectra of cholera toxin and its B protomer produced by the oligosaccharide moiety of the monosialoganglioside G_{M1} were measured as a function of the oligosaccharide concentration. In the presence of oligosaccharide, the spectrum is characterized by three peaks at 282, 288, and 292 nm. A linear increase in difference absorption was observed at these wavelengths vs. oligosaccharide concentration; a saturation effect occurred when the molar ratio of oligosaccharide to cholera toxin was higher than 5. The features of the spectra indicated that the binding with the oligosaccharide affected the environment of tryptophan and tyrosine residues of protomer B. In good agreement with the above results, circular dichroic spectra indicated also a local effect of the binding, mostly restricted to protomer B, while the residues of protomer A remained largely unperturbed. Difference absorption spectra were also measured for cholera toxin in the presence of ganglioside and detergent micelles. The employed gangliosides G_{D1a} and G_{T1b}, unable to bind cholera toxin, interact with the protein by way of contaminating traces of G_{M1}. The preparations of G_{D1a} and G_{T1b} contained 0.8–1.0% (w/w) and 0.4–0.5% (w/w) of G_{M1}, respectively. The results obtained with ganglioside G_{D1a} and G_{T1b} in contrast with the observations made with the oligosaccharide of G_{M1} indicated a major conformational change of the toxin structure. Upon comparison of the conformational change induced by ganglioside micelles with that induced by sodium dodecyl sulfate it may be suggested that the ganglioside micelle, behaving as a detergent, alters the structure of the toxin such as to induce the penetration of protomer A into the lipid milieu. In fact, isolated protomer A, incubated with ganglioside micelles, underwent a structural perturbation similar to that observed with the whole toxin. The complexes between gangliosides and cholera toxin or protomer A were also analyzed electrophoretically after digestion with thermolysin. After interaction with the gangliosides protomer A is partially protected from the attack of the proteolytic enzyme both in the isolated form and in the entire toxin. The specific reduction of the α–γ disulfide bond of protomer A affords complete protection in the intact toxin but not in the isolated protomer A. Therefore, protomer A can enter the ganglioside micelles while the presence of protomer B accelerates this process after reduction of the α–γ disulfide bond. These results suggest a possible role for protomer B in the lag period following the binding of the toxin to the membrane receptors, necessary for the insertion of protomer A into the membrane.

Cholera toxin is composed of two protomers, A and B. The A protomer contains two nonidentical polypeptide chains, α and γ, linked by a single disulfide bridge. The B protomer is believed to be a ringlike pentameric structure made of five identical polypeptide chains noncovalently associated [for general reviews see Finkelstein (1973), Brady & Fishman (1979), and Lai (1980)]. Studies on the binding of cholera toxin to cell surfaces have been pursued in a number of laboratories with the goal of understanding the molecular mechanism of the intoxication process. The intoxication is initiated by a rapid binding of the toxin protomer B to the cell surface monosialoganglioside G_{M1}[1] and is continued by a series of unknown events leading after a lag time to the expression of the catalytic activity of protomer A, which ADP-rybosylates the G regulatory protein of the adenylate cyclase complex. The lag may represent the time necessary for the dissociation of protomer A from protomer B and its subsequent penetration into the cell membrane. It has been concluded from the available evidence that these various processes all depend on initial binding of the toxin to the membrane receptors because binding and intoxication are inhibited specifically by the monosialoganglioside G_{M1} or by its oligosaccharide derivative, oligo-G_{M1} (Cuatrecasas, 1973a,b; Fishman et al., 1978; Richards et al., 1979).

A great deal of attention has, therefore, been paid to the investigation of the molecular aspects of the interaction of cholera toxin with either ganglioside G_{M1} or its derivative oligo-G_{M1}. Evidence has been presented on the number of binding sites per toxin molecule (Fishman et al., 1978) and on the possible positive cooperativity induced by ligand binding (Sattler et al., 1978), as well as on the dynamics of the protein subunit response to the ligand recognition events (Mullin et al., 1976). Several related theories have appeared that propose that the multivalent binding of cholera toxin to five gangliosides G_{M1} produces a change of the protein structure which leads to the membrane insertion of protomer A.

Despite the mechanistic details obtained with these studies the role played by the molecular organization of the ganglioside G_{M1} in cellular membranes and of its interactions with membrane proteins and lipids remains largerly unexplained. However, recent advances have been reported on modifications of the cytoskeleton structure following changes in mobility and distribution of the cell surface G_{M1} receptors induced by the binding (Sahyoun et al., 1981; Hagmann & Fishmann, 1981; Hawkins & Browning, 1982). Accordingly, we have addressed the possibility that the receptor distribution to the binding event may lead to the formation of ganglioside micellar microdo-

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[1] Abbreviations: The ganglioside designation conforms to the nomenclature of Svennerholm (1964) and the IUPAC–IUB Commission on Biochemical Nomenclature (1977). G_{M1}, II^3NeAc-GgOse$_4$Cer; G_{D1a}, IV^3NeuAc,II^3NeuAc-GgOse$_4$Cer; G_{T1b}, IV^3NeuAc,II^3(NeuAc)$_2$-GgOse$_4$Cer; oligo-G_{M1}, II^3NeuAc-GgOse$_4$; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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mains on the plasma membrane (Tomasi et al., 1982). Although this presence has not yet been demonstrated, this hypothesis is particularly appealing since membrane processes such as fusion and transport may be mediated by a transient destabilization of the lipid organization (Cullis et al., 1979; Taraschi et al., 1982). It seemed, therefore, worthwhile to gather more information on the mode of interaction of cholera toxin and isolated protomer A with ganglioside micelles and their oligosaccharide counterpart, the former a simple model of membrane structure and the latter as a case of interaction unaffected by the ganglioside hydrophobic moiety. The results reported herein support the view that the binding with oligo-G_{M1} has almost no effects on protomer A and specific but local effects on protomer B, while ganglioside micelles of G_{D1a} or of G_{T1b}, which are unable to bind cholera toxin (Cuatrecasas, 1973a), grossly alter the overall structure of the protein. A conformational change after incubation with either G_{M1} or G_{D1a} micelles has been demonstrated for isolated protomer A as well. Thus, ganglioside micelles may induce the lipid insertion of protomer A, with protomer B playing a yet undefined role in this process.

Experimental Procedures

Materials. Cholera toxin and protomer B were purified as described by Tomasi et al. (1979). Ganglioside G_{M1} was purified according to Tettamanti et al. (1973). Gangliosides G_{D1a} and G_{T1b} were a generous gift of Fidia Research Laboratories, Abano Terme, Italy. Oligo-G_{M1} was prepared by ozonolysis and alkaline fragmentation of G_{M1} (Ghidoni et al., 1976). Thermolysin (EC 3.4.24.4) and protomer A were purchased from Calbiochem-Behring, San Diego, CA. All other reagents were purchased locally and were of the highest purity available.

Optical Methods. Ultraviolet difference spectra were recorded on a dual-beam Cary 118 recording spectrophotometer as described (Tomasi et al., 1980). The experiments were performed at 25 °C. The toxin and protomer B solutions were prepared at concentrations of either 9×10^{-6} or of 18×10^{-6} M in 0.1 M Tris-HCl buffer, pH 8.2, Protomer A concentration was 2.4×10^{-6} M in the same buffer. Other buffers were also used in a few experiments: (a) 0.1 M Tris-HCl, pH 7.8; b) 0.1 M Tris-HCl, pH 8.8; (c) 0.02 M sodium phosphate, pH 7.8. The oligo-G_{M1} stock solution was 9×10^{-3} M in deionized water. The solutions of G_{M1} (1×10^{-2} M), G_{D1a} (1×10^{-2} M), G_{T1b} (1×10^{-2} M), and $NaDodSO_4$ (3.5×10^{-3} M) in micellar physical form were prepared under standard conditions (Tomasi et al., 1980, 1982).

Circular dichroism spectra were obtained with a JASCO J-41A automatic recording spectropolarimeter. The path length of the cell was 0.1 cm in the range 190–240 nm and 1 cm in the range 240–320 nm. Ellipticity is based on a mean residue weight of 112. The concentrations of cholera toxin and protomer B were calculated from the published values for extinction coefficients and molecular weights (Finkelstein, 1973; Lai, 1980).

Digestion of Cholera Toxin or Protomer A–Micellar Ganglioside Complexes. Cholera toxin (0.36 nmol) was incubated for 60 min at 37 °C in 0.1 mL of 0.1 M Tris-HCl buffer, pH 8.2, with ganglioside (2.5 μmol) G_{M1}, G_{D1a}, and G_{T1b} micelles prepared as described above. Protomer A (0.4 nmol) was incubated under the same conditions with ganglioside (2.5 μmol) G_{M1} and G_{D1a} micelles. When necessary, before or after incubation with gangliosides cholera toxin or protomer A were reduced by adding 1 μmol of dithiothreitol, at 37 °C for 30 min. The reduced and unreduced samples were digested, for 1 h at 40 °C with a 5% (w/w) final concentration of thermolysin, under the conditions described by Matsubara (1970). The reaction was stopped by quickly cooling in ice. The samples were analyzed by $NaDodSO_4$–polyacrylamide disc gel electrophoresis according to the method of Davis (1964) as modified by Laemmli (1970). After electrophoresis the gels were fixed overnight in a mixture of water/methanol/acetic acid (50/40/10 v/v) and stained for 60 min in a solution of 0.1% (w/v) Coomassie blue R250 in the same mixture.

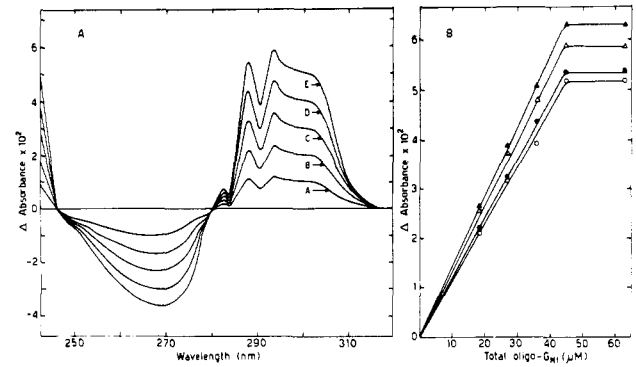

FIGURE 1: Difference spectra of cholera toxin and its protomer B in the presence of oligo-G_{M1}. (A) Difference spectra of cholera toxin (9 μM) generated by oligo-G_{M1} at the following final concentrations: A, 9 μM; B, 18 μM; C, 27 μM; D, 36 μM; E, 45 μM. (B) Plots of the absorbance difference values for cholera toxin (○, △) and its protomer B (●, ▲) at 288 and 292 nm, respectively, as a function of oligo-G_{M1} concentration; the concentration of the two proteins was 9 μM.

Results

Ultraviolet Difference Spectra of Cholera Toxin and Protomer B Produced by Oligo-G_{M1}. Figure 1A shows typical difference spectra of cholera toxin produced by oligo-G_{M1}. The spectrum characterized by three peaks at 282, 288, and 292 nm and a shoulder at 300 nm was enhanced in intensity with increasing oligo-G_{M1} concentrations. It is apparent that the perturbations become less intense with the increase in the amount of oligo-G_{M1}, reaching a plateau at a toxin–ligand molar ratio of 5.8. The spectrum features generated by oligo-G_{M1} can be taken as indicative of an involvement of tryptophan and tyrosine residues on the binding. Considerable attention has been paid previously to the role of the lone tryptophan residue of the β-polypeptide chain in the interaction of cholera toxin with the G_{M1} receptor (Sillerud et al., 1981; De Wolf et al., 1981a,b). The difference spectra obtained (283–288 nm) do suggest that oligo-G_{M1} interacts with a site on the cholera toxin molecule affecting also tyrosine residues.

The above results were also confirmed when the binding of protomer B alone with oligo-G_{M1} was studied under the same experimental procedures. In these experiments, almost identical spectra (not shown) were obtained at similar protein–ligand molar ratios, the only appreciable difference being a small increase in the intensity of the 288- and 292-nm peaks. Since complex formation between either cholera toxin or its B protomer with the oligosaccharide moiety of G_{M1} generates the same difference spectra, binding has no effect on the aromatic side-chain chromophores of protomer A. The linear dependence of the intensities of the peaks at 288 and 292 nm from oligo-G_{M1} concentration both for cholera toxin and protomer B is shown in Figure 1B. The same linearity was also observed in initial velocity studies, measuring the increase in absorbance at 292 nm as a function of time. All these data seem to suggest a substantial uncooperative effect of the binding, in contrast to the evidence reported by Sattler et al.

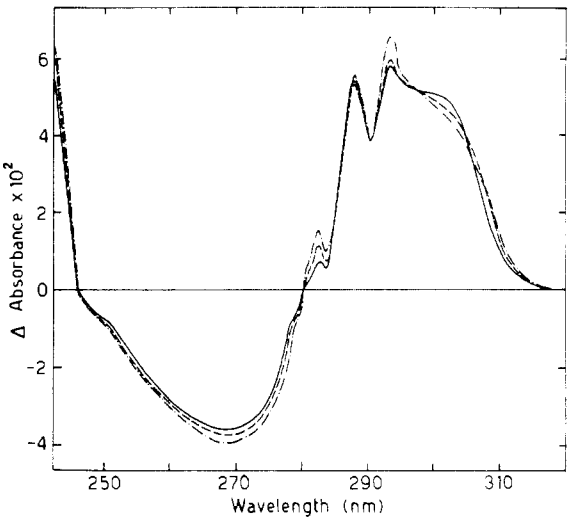

FIGURE 2: Effect of pH on the difference spectrum of cholera toxin generated by oligo-G_{M1}. Cholera toxin and oligo-G_{M1} concentrations were 9×10^{-6} and 4.5×10^{-3} M, respectively, in (—) 0.1 M Tris-HCl buffer, pH 7.8, (---) 0.1 M Tris-HCl buffer, pH 8.2, and (·--) 0.1 M Tris-HCl buffer, pH 8.8.

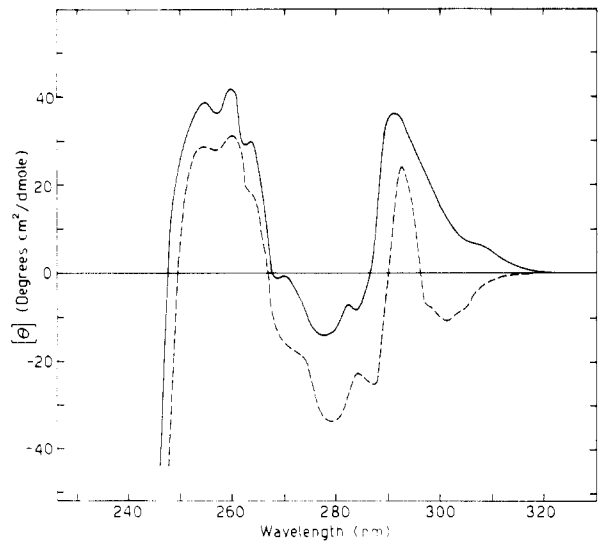

FIGURE 3: Circular dichroic spectrum of cholera toxin. The spectrum of cholera toxin (9 μM) was recorded without (—) and with (---) 45 μM oligo-G_{M1}.

(1978) using a more sensitive method, i.e., direct binding of labeled oligo-G_{M1} to cholera toxin, favoring a small positive cooperativity of protomer B subunits upon ligand binding.

The effect of changes in the electrostatic environment of the chromophores exposed by the binding with oligo-G_{M1} is shown in Figure 2. It is well-known that electrostatic effects influence the absorption characteristics of the phenolic and indolic chromophores (Donovan, 1973). Accordingly our experiments reveal significant increasing of the maxima at 282, 288, and 294 nm with increasing pH, while a blue shift is observed for the shoulder at 300 nm. These results, similar to those obtained with model compounds when the phenolic and indolic groups become more positively charged (Donovan, 1973), provide additional information on the involvement of tyrosine residues in the binding of cholera toxin to oligo-G_{M1}.

Circular Dichroism Spectra of Cholera Toxin and Protomer B: Effects of Oligo-G_{M1}. Figure 3 presents the circular dichroism spectrum of cholera toxin. The spectrum is characterized by one major positive band at 294 nm and a less resolved broad peak between 255 and 265 nm. It has a negative through at 278 nm. Repeated measurements have shown these

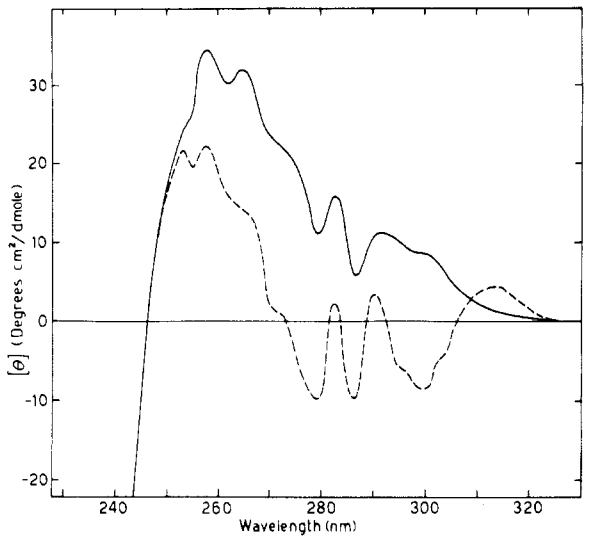

FIGURE 4: Circular dichroic spectrum of protomer B of cholera toxin. The spectrum of protomer B (9 μM) was recorded without (—) and with (---) 45 μM oligo-G_{M1}.

bands and the crossovers to be quite reproducible. The circular dichroic spectrum of cholera toxin bound to oligo-G_{M1} was also investigated. The spectra in the far-ultraviolet peptide region are not shown since they were quite similar to those of the native toxin and almost undistinguishable from those already reported (Fishman et al., 1978; Roda et al., 1977). The spectrum of the toxin bound to oligo-G_{M1} was, instead, considerably different from that of the native protein. The tryptophan band is hidden, and the spectrum of the ligand-bound toxin is characterized by a negative Cotton effect at 296 nm. Of interest was also the observation of the increased magnitude of the tyrosine circular dichroic band in the presence of oligo-G_{M1}. Thus, tyrosine residue perturbation is clearly associated with the binding of oligo-G_{M1} to cholera toxin.

Figure 4 shows the circular dichroic spectra of protomer B. The presence of oligo-G_{M1} generates the same spectral pattern, observed with whole cholera toxin, in the region of the tryptophan band. However, in the region of the tyrosine bands, the structure of protomer B is more profoundly affected than the structure of the whole toxin by the presence of the ligand. The ellipticity value of the ligand-bound protomer B changes to a negative band at 275 nm, suggesting that the bound ligand either affects the charge of the involved tyrosine residues or locks them in a more asymmetric configuration (Schechter et al., 1971). The comparison of the results obtained with cholera toxin and protomer B shows that in the whole toxin the tyrosine residues of protomer A are largely unperturbed by the binding while exerting a masking effect on the circular dichroic bands of the chromophores of protomer B affected by the binding.

Ultraviolet Difference Spectra of Cholera Toxin and Protomer A Produced by Micellar Gangliosides. The direct evidence of the interaction of cholera toxin with ganglioside G_{M1} in micellar physical form cannot be obtained by the difference spectroscopy technique, since the toxin immediately cross-links the G_{M1} micelles as revealed by the appearance of turbidity upon mixing the toxin and G_{M1} solutions. We have, therefore, resorted to an indirect approach, i.e., the study of interaction of cholera toxin with gangliosides G_{D1a} and G_{T1b}, which possess carbohydrate moieties unable to bind cholera toxin (Cuatrecasas, 1973a), and of the interaction of protomer A with gangliosides G_{M1} and G_{D1a}. Preliminary experiments had shown that G_{D1a} contained small amounts of G_{M1}. This

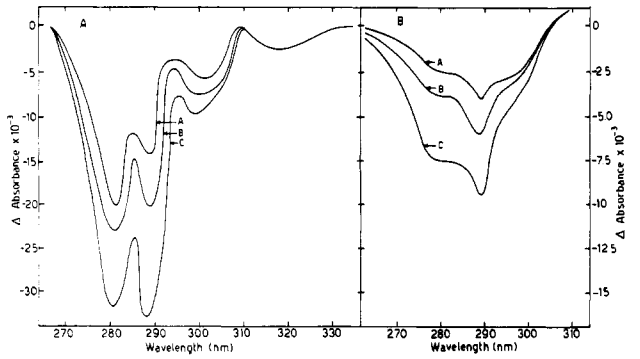

FIGURE 5: Difference spectra of cholera toxin in the presence of gangliosides G_{D1a} and G_{T1b} in micellar form. Cholera toxin (9 μM) was mixed with either 7×10^{-3} M G_{D1a} (A) or 7×10^{-3} M G_{T1b} (B), and the spectrophotometer was compensated as described in the text. The spectra were subsequently recorded at different time intervals: A, 30 min; B, 60 min; C, 120 min.

amount,[2] approximately 0.8–1% on a weight basis, was sufficient for binding cholera toxin without any detectable turbidity and originating an identical spectral profile to that obtained with oligo-G_{M1}. Thus, the interaction of cholera toxin with the binding sites of the oligosaccharide moiety of G_{M1} specifically modifies the environment of the tyrosine and tryptophan residues of the binding subunits. At variance with results obtained with oligo-G_{M1}, the interaction of cholera toxin with micellar ganglioside did not show saturation kinetics. Of interest, in fact, was the observation of a significant change in the spectrum intensities with respect to time. Presumably, the hydrophobic portion of the ganglioside plays an additional role in the interaction with cholera toxin. In order to evaluate this role, after mixing toxin and ganglioside solutions and recording the difference spectrum, the spectrophotometer was compensated to give a satisfactorily straight base line, or "zero line", on the G_{M1}–toxin complex, in the wavelength region of interest. The spectra recorded thereafter have intensities that are clearly time dependent (Figure 5A). Incubation with ganglioside G_{D1a} micelles induces a slow but remarkable change in the difference spectrum of cholera toxin. The spectrum has a through at 287 nm, indicating a contribution of aromatic side-chain blue shifts to the unfolding of the protein (Steinhardt et al., 1972). When G_{D1a} micelles were preincubated with protomer B for saturating the contaminant G_{M1}, prior to the addition of cholera toxin, similar spectra of much lower intensities were recorded which suggested that protomer A is principally affected by this low process. The presence of the bulky protomer B on the micelle surface prevents the subsequent binding of the native toxin and therefore the hydrophobic interaction of protomer A with the ganglioside micelle (Tomasi et al., 1982).

Figure 5B shows the spectra obtained with micelles of G_{T1b}, which contains less than 0.5% of G_{M1}. The spectra indicate that the contribution of G_{T1b} micelles to the unfolding of the bound cholera toxin is smaller and slower than that of G_{D1a} micelles under the same experimental conditions. These results cannot be due only to a lower amount of cholera toxin bound

[2] The amount of G_{M1} present in G_{D1a} micelles was determined empirically by adding increasing amounts of protomer B to a fixed amount of G_{D1a} micelles and, immediately after mixing, recording the increase in difference absorption intensity at 292 nm. A saturation curve was obtained from which, assuming that each molecule of protomer B had bound five molecules of ganglioside G_{M1}, it was calculated that 0.8–1.0% (w/w) of G_{M1} was present in the G_{D1a} preparation employed. Similar experiments with G_{T1b} gave a lower level of contamination by G_{M1} of the order of 0.4–0.5% (w/w).

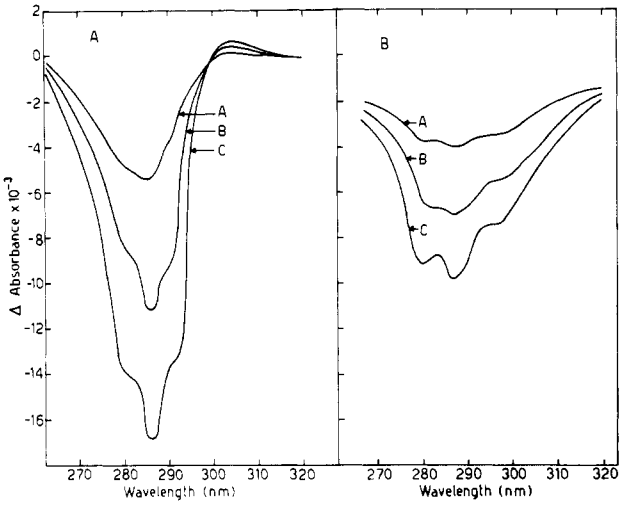

FIGURE 6: Difference spectra of protomer A in the presence of gangliosides G_{M1} and G_{D1a} in micellar form. Protomer A (2.4 μM) was mixed with either 4.0×10^{-3} M G_{M1} (A) or 4.0×10^{-3} M G_{D1a} (B), and the spectra were recorded at different time intervals: A, 30 min; B, 60 min; C, 120 min.

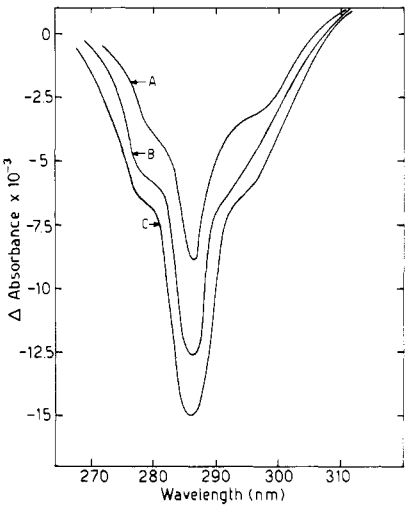

FIGURE 7: Difference spectra of cholera toxin in the presence of $NaDodSO_4$. Cholera toxin (9 μM) was mixed with 3.5×10^{-3} M $NaDodSO_4$, and the spectra were recorded at different time intervals: A, 30 min; B, 60 min; C, 120 min.

to the G_{M1} impurities of G_{T1b} micelles as compared to G_{D1a} micelles, since the spectra obtained with the two gangliosides have significantly different features in the regions of 280 and 294 nm. A more likely explanation may reside in the different structure and aggregation properties of the two gangliosides. The differences in polarity and ionic character between G_{D1a} and G_{T1b} contributed by the number of sialic acid residues are correspondingly paralleled by differences in the 18-carbon to 20-carbon sphingosine ratio (Yohe et al., 1976).

The increase in sialic acid content decreases the micellar size from 230 for G_{D1a} to 120 monomers per micelle for the G_{T1b} (Yohe et al., 1976; Corti et al., 1980). Differences in molecular structure and in micelle size and shape affect also the mode of interaction of other micelle-forming amphiphiles with water-soluble proteins (Tanford & Reynolds, 1976).

Figure 6 shows the result of the interaction between purified protomer A and micelles of ganglioside G_{M1} and G_{D1a}. Ganglioside G_{M1} micelles induce a time-dependent conformational change of the protein structure more pronounced than that observed with G_{D1a} micelles. The balance of the hydrophobic and polar characteristics probably makes ganglioside G_{M1}, in its mode of interaction with proteins, more similar to

$NaDodSO_4$ than to other gangliosides. In fact, the unfolding of the protomer A structure in the presence of G_{M1} is quite similar to that observed with the whole toxin in the presence of $NaDodSO_4$ (Figure 7) and also similar to the same process induced on other proteins such as bovine serum albumin either by G_{M1} (Tomasi et al., 1980) or by $NaDodSO_4$ (Bigelow & Sonenberg, 1962). However, the magnitude of the effects induced on protomer A is less pronounced than that observed with the whole toxin.

Effect of $NaDodSO_4$ on the Difference Spectrum of Cholera Toxin. As seen from Figure 7, the difference spectra generated by the interaction of cholera toxin with $NaDodSO_4$ micelles are typical tyrosine difference spectra. They have a large minimum at 287 nm and a smaller one at 280 nm and are indicative of a disruption of the secondary and tertiary structure of the protein (Bigelow & Sonenberg, 1962).

The observation reported above, correlated with data obtained with other techniques (Tomasi et al., 1982), suggests that the ganglioside and detergent micelles interact with cholera toxin in a dissimilar way. The conformational change induced by $NaDodSO_4$ affects the whole protein structure, while the gangliosides affect mostly protomer A.

Effect of Ganglioside Micelles on Cholera Toxin and Protomer A Digestion by Thermolysin. In order to verify which toxin domain is affected by the slow conformational change induced by ganglioside micelles, we have digested the ganglioside-bound toxin with thermolysin. Parts A and B of Figure 8 show that the α chain of cholera toxin is preferentially hydrolyzed, while protomer B is almost unaffected by the proteolytic enzyme. Moreover, thermolysin completely digested the α chain when the disulfide bridge, which holds together the two functional regions α and $\gamma\beta_5$, was reduced. Thermolysin, which has a specificity toward peptide bonds made of hydrophobic residues (Pank et al., 1982), seemed, therefore, a suitable tool for analyzing the interactions of cholera toxin and of protomer A with micellar gangliosides. When cholera toxin was preincubated with G_{M1} micelles prior to the digestion with thermolysin, protomer A was trapped at the top of the gel in the high molecular aggregates formed by the multivalent protomer B and ganglioside micelles (Staerk et al., 1974). In fact, when G_{M1}-bound toxin was reduced after digestion, approximately 30–60% of the α chain was recovered. Furthermore an almost identical protection was observed with isolated protomer A, and this result was not affected by reduction prior or after digestion. All these similar results are summarized in Figure 8C where only the amount of α chain recovered from protomer A, preincubated with ganglioside G_{M1} micelles, and then reduced is shown (dashed line). In all these experiments the amount of α chain shielded by the ganglioside micelles from proteolysis roughly increased with the incubation time. However, a direct correlation between the time courses of the optical changes and of the resistance to the thermolysin attack cannot be made because the two methods probably detect different protein domains and have different sensitivities toward the dynamics of the process under study.

Interestingly, the α chain was completely protected from digestion when the toxin was reduced before the attack of the proteolytic enzyme (Figure 8D). Moreover, the micelles of G_{D1a} or G_{T1b} are at least as efficient as G_{M1} micelles in protecting the α chain from the attack of the proteolytic enzyme (Figure 8E,F). Thus, protomer B has a protective effect on the chain additive to that of the ganglioside micelles. This additional role of protomer B, evidenced only when the α chain is released by reduction prior to the digestion, is difficult to distinguish from the direct action of the ganglioside micelles

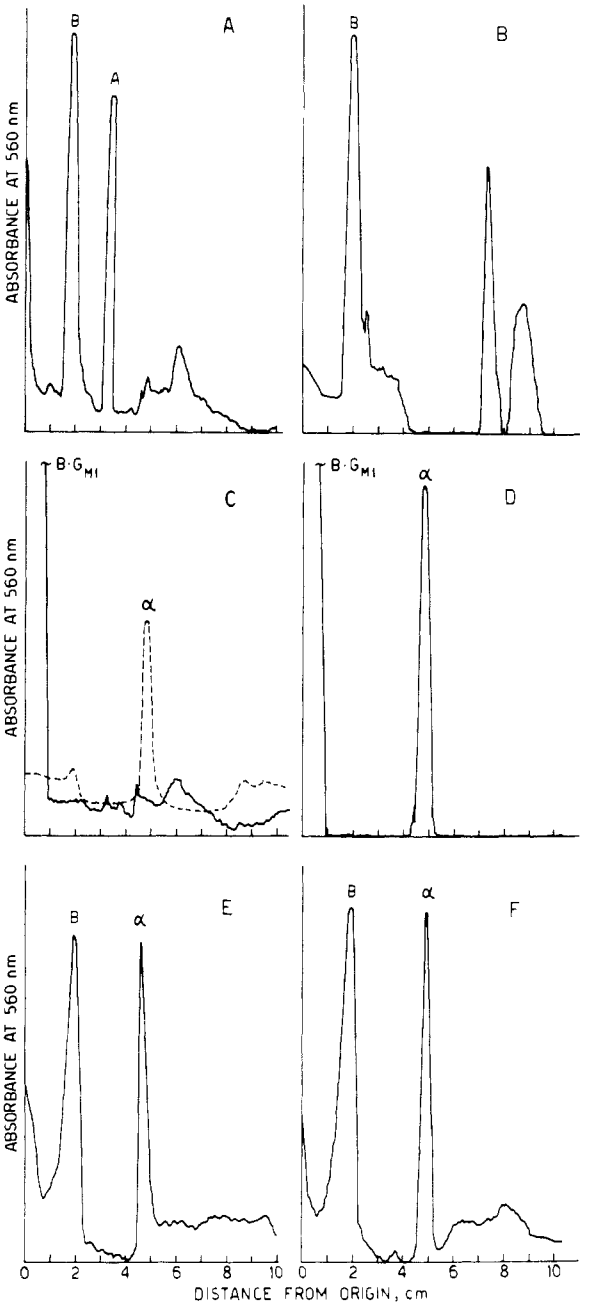

FIGURE 8: $NaDodSO_4$–polyacrylamide gel electrophoresis of thermolytic digestion mixtures of cholera toxin or protomer A preincubated with different gangliosides. The densitometric scan of the Coomassie blue stained gels was obtained after preincubation and digestion of the toxin as described under Experimental Procedures and electrophoresis on a 20% $NaDodSO_4$–polyacrylamide gel run according to Laemmli (1970). (A) Cholera toxin; (B) cholera toxin reduced with dithiothreitol; (C) cholera toxin preincubated with G_{M1} (continuous line) or protomer A reduced with dithiothreitol and incubated with G_{M1} (dashed line); (D) cholera toxin preincubated with G_{M1} and reduced with dithiothreitol; (E) cholera toxin preincubated with G_{D1a} and reduced with dithiothreitol; (F) cholera toxin preincubated with G_{T1b} and reduced with dithiothreitol.

themselves. Nevertheless, all these results show that the interaction with micellar gangliosides of protomer A, both in isolated form or in the entire toxin, is slower than that observed with its α-chain component, released by reduction, in the presence of protomer B interacting with its membrane receptor.

Discussion

The results of spectroscopic studies demonstrate that the interaction of cholera toxin with oligo-G_{M1} induces detectable alterations in the ultraviolet absorption and circular dichroism

spectra of the protein. These changes are characterized by a perturbation of the protein structure in the neighborhood of some of its aromatic amino acids and suggest that tryptophan and tyrosine residues are mostly affected by the binding. Investigations on protomer B, saturated with the ligand, gave strikingly similar results to those obtained with the whole toxin, thus indicating that the binding itself is not sufficient for generating difference spectra of the residues of protomer A and that only the aromatic residues of protomer B became exposed as a consequence of the interaction with oligo-G_{M1}. Our results, confirming the reported involvement of the lone tryptophan residue of the β-polypeptide chain in the binding (De Wolf et al., 1981a,b; Sillerud et al., 1981), demonstrate for the first time also the participation of the β-chain tyrosyl residues in the toxin–ligand interaction.

The spectra generated upon binding do not necessarily represent a conformational change of the protein structure. Moreover, the kinetic analysis of saturation curves suggests that the oligo-G_{M1} binding sites are apparently noninteracting. This is in contrast with the reported positive cooperativity for the binding of oligo-G_{M1} to cholera toxin when the binding was measured by equilibrium dialysis (Sattler et al., 1978). The discrepancy of these results is probably coincidental. It should be emphasized, in fact, that UV difference spectroscopy is less sensitive than the direct measurement of binding with labeled ligand and that Sattler et al. (1978) had to utilize a toxin molecular model, made of four interacting and one noninteracting polypeptide chain, as a tentative working hypothesis for positive cooperativity. Attempts to equate binding measurements with molecular models should be done when a more precise description of the toxin structure becomes available.

In regard to the possibility that oligo-G_{M1} binding induces a conformational change of the protein structure, the circular dichroic spectra provide interesting information. Since major changes in circular dichroic spectra upon binding were observed only in the aromatic side-chain region, the spectra can arise only from a combination of both direct shielding of these groups from the solvent and small changes involving side-chain reorientation without any effect on the polypeptide backbone. The perturbation of the toxin structure observed in the far-ultraviolet region was too small (Fishman et al., 1978) to give any evidence of a conformational change of the toxin after binding.

The circular dichroic spectra of both cholera toxin and protomer B further define the role of the β-chain tyrosyl residues in the binding of oligo-G_{M1}. The β-polypeptide chain contains three tyrosine residues located at positions 12, 27, and 76 of its 103 amino acid sequence (Lai, 1980). The predicted secondary structure of the binding subunit suggests that residues 27 and 76 are on β-pleated sheet structures while residue 12 is located on a β turn of the molecule (Duffy & Lai, 1979). The tyrosine residue at position 12 is close to the disulfide bridge of the β chain and almost equally spaced from the lone tryptophan residue (De Wolf et al., 1981a,b) and the arginine at position 35 (Duffy & Lai, 1979), both of which are involved in the interaction of the binding subunit with ganglioside G_{M1}. However, the experiments therein indicate only that the tyrosine residue(s) of the β-polypeptide chain are highly accessible to the oligosaccharide and participate in the binding process. In sum, a picture emerges of other structural features of cholera toxin, which have not been previously addressed, important in its interaction with the ligand.

The interaction between cholera toxin and ganglioside micelles gave an interesting picture. While with ganglioside G_{M1} the perturbing effect of the oligosaccharide moiety masked the hydrophobic effect of the lipid portion of the gangliosides, experiments with other gangliosides, unable per se to bind the toxin, gave spectral differences somehow similar to those observed in the interaction of bovine serum albumin with ganglioside G_{M1} micelles (Tomasi et al., 1980). The latter interaction is mostly hydrophobic in nature, and the same holds true for cholera toxin as shown by the striking changes in the spectral regions concerning the aromatic amino acids. We had previously demonstrated with the hydrophobic photolabeling technique that of the entire toxin only the α chain showed a marked preference for being in a hydrophobic environment (Tomasi & Montecucco, 1981; Hagman & Fishman, 1981; Tomasi et al., 1982). This is now confirmed by the digestion experiments which show that the α chain can be shielded by the ganglioside micelle from the thermolysin attack. Moreover, the α chain can enter the micelles without being previously released from protomer A by reducing agents as shown by the optical and digestion experiments. However, the complete entrapment of the α chain into the ganglioside micelles occurs only in the presence of protomer B.

Micellar gangliosides containing trace amounts of the G_{M1} receptor represent a model for studying the interaction of the water-soluble cholera toxin with a lipid membrane organized in a nonbilayer structure. Recently it has been suggested (Cullis & De Kruijff, 1979; Hui et al., 1981) that membrane processes such as fusion and transport may be mediated by the transient formation of microenvironments organized not in a bilayer but in a micellar-like structure. Although there is no evidence for the presence of ganglioside micellar microdomains on the plasma membrane, the results presented here show that the interaction of cholera toxin with the model ganglioside micelles is a two-step process quite similar to that observed with cell membranes. A very fast reaction allows the toxin to recognize the oligosaccharide portion of the contaminating G_{M1} without any major change of the protein conformation by means of subtle rearrangements of amino acid side chains. The side-chain reorientation involves at least the lone tryptophan, arginine at position 35, and tyrosine residue(s) of the β-polypeptide chain. After the binding, a slower process takes place, during which the ganglioside micelle, behaving as a detergent, grossly alters the structure of the toxin embedding protomer A in the lipid milieu. If during this process the enzymatically active α chain is somehow released from protomer A, protomer B will play an additional role in the penetration of the α chain in the hydrophobic core of the membrane. This model is consistent with the tight, essentially irreversible binding of protomer B to the cell surface receptors and with the following lag period, necessary for the insertion of the active protomer A into the membrane.

Registry No. G_{M1}, 52930-43-5; G_{D1A}, 12707-58-3; G_{T1B}, 59247-13-1.

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