DISSOCIATION OF CHOLERA TOXIN FUNCTIONAL REGIONS AFTER INTERACTION WITH VESICLES CONTAINING GANGLIOSIDE G_{M1}

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1. Introduction

The toxin, produced by *Vibrio cholerae*, is an 84 000 mol. wt protein [1], composed of three unique polypeptide chains α, β, γ and has a molecular formula $\alpha\gamma\beta_5$ [2–4]. The α and γ chains are linked through a single disulfide bond, forming protomer A. Five identical polypeptide chains form a stable aggregate, protomer B. Protomer A and B are very tightly bound and can be dissociated slowly with detergents. Treatment of the toxin with dithiothreitol gives selective reduction of the disulfide bond linking two distinct functional regions, fragment α and fragment $\gamma\beta_5$ [5,6]. Although devoid of any catalytic activity fragment $\gamma\beta_5$ nevertheless retains the ability of the intact toxin to recognize the cell surface receptor, the monosialoganglioside G_{M1}. Exposure to the solvent of the catalitically active region of the α chain, after reduction, enables it to stimulate adenylate cyclase.

The time course of activation of adenylate cyclase in intact relative to broken cells has shown that there is a lag period between the initial binding step of the toxin to intact cells and the final cyclase activation step [7–9]. Studies with fluorescent probes and immunoelectron microscopy have provided evidence that the latent period may represent the time required for a redistribution of the toxin–receptor complexes in the cell membrane [10,11]. Whether the active functional region penetrates the membrane through channels [12] or a change in the conformation of the toxin [13], resulting from the mobility of the toxin–G_{M1} complexes in the membrane, remains to be determined. At present, although some authors have reported an interaction of hydrophobic nature between protomer A and membranes [13,14], no direct evidence of the dissociation either of protomer A or of the α chain has been obtained, after binding of the toxin to biological membranes. In view of these observations, we examined the characteristic and specific binding of cholera toxin to vesicles containing G_{M1} [15] with respect to the possible interactions of the active α fragment with model membranes. The effect of binding on the reactivity of the interchain disulfide bond connecting the α and $\gamma\beta_5$ functional regions was also studied.

This communication shows that the interaction of cholera toxin with G_{M1}–vesicles is strong enough to induce perturbations in the lipid membrane but it does not affect the dissociation of the active α chain from the vesicle-bound toxin.

2. Materials and methods

Toxin was isolated as in [16]. Gangliosides G_{M1} and G_{D1a} were isolated by the method in [17] and were >98% pure. Oligo-G_{M1}, the oligosaccharide moiety of G_{M1}, was prepared by ozonolysis and

Abbreviations: G_{M1}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; oligo-G_{M1}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucose

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alkaline fragmentation [18,19]. A constant amount of G_{M1} and the amounts of phosphatidylcholine (egg yolk, Supelchem) necessary to give the G_{M1}–lipid ratios R 0.108 and 0.228 were utilized. Vesicles were prepared according to the method in [20], isolated after gel filtration on a Sepharose 4B column (1.5 × 100 cm) and used throughout our experiments. The amount of ganglioside exposed to the solvent, on the outer-vesicle surface was determined by estimating *N*-acetylneuraminic acid [17] released after treatment of vesicles, prepared with G_{D1a}, with *Vibrio cholerae* neuraminidase (Serva, Heidelberg) and confirmed by the method in [20]. Vesicles were found to have mol. wt $2.3 \pm 0.3 \times 10^6$ and 60% of G_{M1}, available for binding, exposed on the outer-vesicle surface. Binding was assayed by incubating the amounts of vesicles indicated in fig.1 with cholera toxin (0.12 nmol) in 0.02 M Tris–HCl buffer (pH 8.2), 0.002 M EDTA, at 30°C, for 5 min, in 0.1 ml final vol. For inhibition experiments the same amount of toxin was incubated at 30°C, with the amount of vesicles containing 6.4 nmol G_{M1} on the outer-vesicle surface prior to the addition of 84 nmol oligo-G_{M1}. Samples were analyzed by polyacrylamide disc-gel electrophoresis as in [5]. The bound toxin does not enter the gel. The unbound toxin was quantitated as in [5] and subtracted from the amount added to give the amount bound to vesicles. Non-specific binding was determined by incubating cholera toxin with G_{M1}-free vesicles. In control experiments identical amounts of unbound toxin were also recovered from the supernatant after centrifugation of the incubation mixtures at 250 000 × *g* for 3 h.

Spectral measurements were made with the use of Yankeelow double-sector cells. The sample cell contained 1 ml vesicle dispersion in the first compartment and 1 ml cholera toxin (2.4×10^{-6} M) in the second compartment. The reference cell contained in the respective compartments the vesicle dispersion and the solvent buffer. Vesicle aggregation was measured by recording the turbidity of the vesicle–toxin mixtures, immediately after mixing of the double-sector cells, at 30°C and 400 nm, in a Varian Cary 118 recording spectrophotometer.

Similar results for the binding experiments were also obtained with vesicles containing the amounts of G_{M1} and cholesterol described in [15] and are, therefore, not reported.

3. Results and discussion

Figure 1 shows the results of experiments in which cholera toxin was added to vesicles with different G_{M1}–lipid molar ratios. Since all the toxin was recovered in the supernatant in the case of vesicles without G_{M1}, in the conditions employed there was no significant entrapment of the toxin. At saturation each toxin molecule binds 1.7 and 5.3 molecules of membrane-bound G_{M1}, for R = 0.108 and 0.228, respectively. Apparently, a different localization of the ganglioside receptors less widely spaced, at higher G_{M1}–lipid ratios, results in a lower binding affinity.

When toxin was added to G_{M1}–vesicles, the absorbance increase, observed with longer incubation times, is presumably due to the formation of vesicle aggregates (fig.2), which resembles cell agglutination induced by lectins [21]. All the toxin added is bound to the vesicles within 5 min, while there is still no significant aggregation. The rate at which turbidity increased was dependent on ganglioside concentration on the outer-vesicle surface and no aggregation was observed, under the same experimental conditions, with vesicles free of G_{M1}.

It has been shown that cholera toxin contains multiple binding sites (5–6) for the oligosaccharide

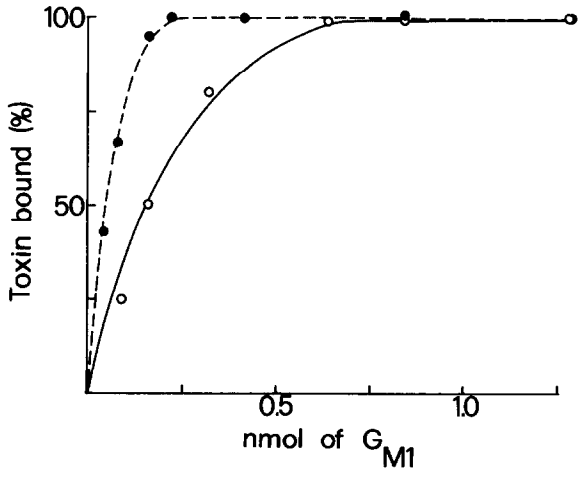

Fig.1. Binding of cholera toxin to G_{M1}–vesicles. Binding experiments were performed as in section 2. Results are expressed as percentage of protein bound. In the abscissa the amounts of G_{M1} present on the outer-vesicle surface are reported. (•– – –•) R = 0.108; (o——o) R = 0.228.

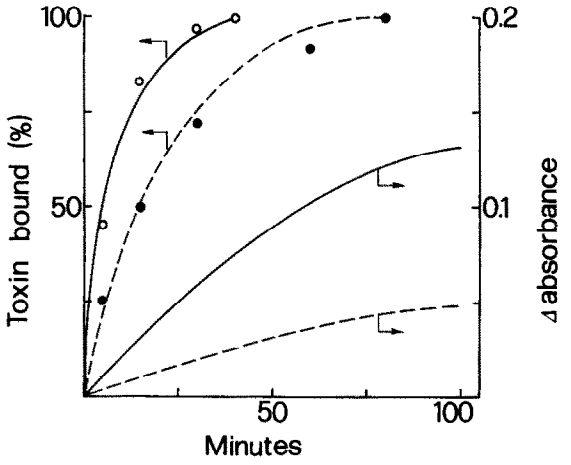

Fig.2. Kinetics of aggregation of G_{M1}–vesicles after addition of cholera toxin and reversibility of the binding in the presence of oligo-G_{M1}. Aggregation was measured from the increase in turbidity at 400 nm. (– – –) $R = 0.108$; (——) $R = 0.228$. For the inhibition experiments cholera toxin was incubated with G_{M1}–vesicles for the times indicated prior to the addition of oligo-G_{M1}. After 5 min, the mixture was analyzed as described in fig.1. (•– – –•) $R = 0.108$; (○——○) $R = 0.228$.

moiety of ganglioside G_{M1} [22]. At higher G_{M1}–lipid ratios, the density of the oligosaccharide moieties protruding out of the vesicle surface introduces steric constraints which will not allow the receptors of a single vesicle to become correctly positioned with respect to the toxin. Such a high density results in the formation of preferential bindings between the toxin and the receptors of different vesicles. At lower G_{M1}–lipid ratios all 5 toxin binding sites are preferentially bound to the receptors of a single vesicle. The data of fig.1 appear to support this possibility; decreasing by 50% the density of the ganglioside on the vesicle surface allows each toxin molecule to bind 5 molecules of G_{M1} instead of 2.

When aggregation occurs, addition of specific inhibitors, such as oligo-G_{M1}, reverses the process only in the early stages (fig.2). The reason for this may be that all toxin does not bind to G_{M1} in an equivalent manner since the time course of the binding process may be mediated by a different localization of G_{M1} in the membrane. A change in the topological distribution of receptor sites may be expected since it has been reported that cell-bound toxin diffuses both laterally and vertically in the membrane, after incubation for times comparable to those of our aggregation experiments [11]. Moreover the irreversibility of the binding at longer incubation times supports a mechanism similar to the fusion process described for the interaction of lectins with liposomes [23]. No matter what kind of process takes place at the membrane, after toxin binding, either a diffusion of the toxin in the lipid matrix or a vesicle fusion, the subsequent structural or topological rearrangements may alter the reactivity of the interchain disulfide bond connecting cholera toxin functional regions.

The kinetics of reduction of cholera toxin bound to G_{M1}–vesicles is shown in fig.3. Comparison of the rates of reduction, under the same conditions, with free cholera toxin shows no effect of the binding to the model membranes on the reactivity of the disulfide bond. Although the lipid environment of the membrane affords partial protection against the characteristic dissociation–aggregation transition of the α chain [5,6], there was neither a specific interaction between the α chain and the membrane lipids, nor a diffusion

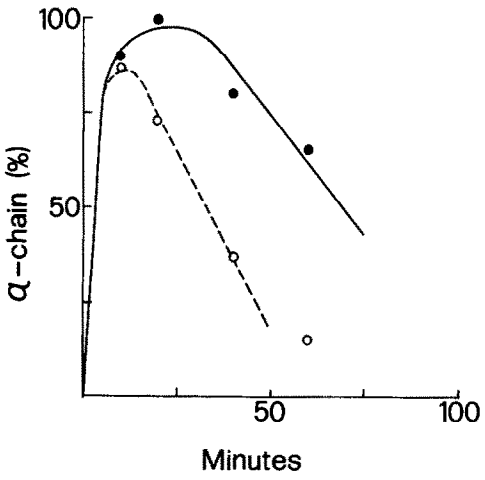

Fig.3. Dissociation of the α chain from reduced cholera toxin bound to G_{M1}–vesicles. Cholera toxin (0.24 nmol) was incubated for 1 h at 37°C with an amount of vesicles containing 5.5 nmol G_{M1} on the outer-vesicle surface. In control experiments the addition of vesicles was omitted. After the addition of 1 μmol dithiothreitol, aliquots of the samples were withdrawn and analyzed as in [5]. Results are expressed as percentage of the chain recovered from reduced samples. (○– – –○) cholera toxin; (•——•) cholera toxin plus vesicles.

of the active region itself into the vesicle membrane. In fact, the α chain can be quantitatively recovered also from fully aggregated toxin–vesicle complexes, treated with dithiothreitol after several days or weeks of incubation.

The results described here suggest that although G_{M1} may be an important part of the receptor for cholera toxin, it is not sufficient simply for G_{M1} to be present in the membrane, because when the toxin becomes essentially irreversibly attached to the membrane, the functional region can be still quantitatively removed by reduction. Thus, the α active functional region seems to remain on the outside of the vesicle membrane, exposed to the solvent, and other factors or regions of specificity may be involved in its penetration.

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