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INTERACTION OF SHIGELLA TOXIN WITH GLOBOTRIAOSYL CERAMIDE RECEPTOR - CONTAINING MEMBRANES: A FLUORESCENCE STUDY*

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Summary: The interaction of the B-subunit of Shigella toxin with a globotriaosyl ceramide receptor incorporated into phosphatidylcholine vesicles was studied by fluorescence spectroscopy. From the position of the maximum in the emission spectrum and the accessibility to acrylamide quenching, it is concluded that a single trytophan of a free B-chain is located in a highly polar environment, most likely on the surface of the folded polypeptide chain. Binding of B-subunits to the membrane-associated globotriaosyl ceramide results in a strong enhancement of fluorescence intensity and a small blue-shift of the emission maximum; these effects suggest a conformational change in the protein which provides a new environment to a tryptophan residue. However, the polarity of this new environment is still relatively high - as indicated by the position of the emission maximum at 344 nm - and suggests that the receptor-bound B-chain remains largely on the membrane surface, without penetrating the hydrophobic interior of a lipid bilayer. On the other hand, the A-chains are shown to interact directly with the receptor-free lipid bilayers; this nonspecific interaction may play a role in the mechanism by which A-subunit traverses the membrane of a target cell.

Shigella toxin, a potent cytotoxic protein produced by Shigella dysenteriae, has been implicated in a number of pathophysiological conditions including infectious diarrhea and hemolytic-uremic syndrome (1-3). Recent structural studies have revealed that the toxin consists of an A chain of MW 32,000 that is noncovalently complexed with five B chains of MW 7,700 (4-6). This structure is similar to that of other bacterial toxins such as cholera toxin and E. coli heat-labile toxin in which the functions served by the A and B subunits are complementary (7,8). The toxic activity of Shigella toxin is associated with the enzymatically active A subunit which inhibits protein synthesis by cleaving a N-glycosidic bond of the 28 S ribosomal RNA (9-11). The function of the B subunit on the other hand, is to bind to a cell membrane receptor (5,12). A glycolipid receptor for Shigella toxin has been recently purified from HeLa cells and from rabbit jejunum and identified as

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globotriaosyl ceramide (13). Specific binding of the toxin has been also reported for other glycolypids containing the disaccharide Gal(α1-4)Gal β (14).

Despite remarkable progress in the biochemical characterization of Shigella toxin and its glycolipid receptor, the molecular aspect of the toxin-receptor interaction, as well as the mechanism by which the toxin (or its enzymatically active subunit) penetrates the membrane barrier and enters the cell, remain unknown. In this communication, we describe studies using fluorescence spectroscopy, aimed at elucidating the biophysical mechanisms of interaction between Shigella toxin and model membranes containing the glycolipid receptor globotriaosyl ceramide.

MATERIAL AND METHODS

Globotriaosyl ceramide [Gal(α1-4)Gal(β1-4)Glc-Cer] was purchased from Supelco Inc., dimyristoylphosphatidylcholine (DMPC) was from Avanti Polar Lipids and acrylamide from Bio-Rad Laboratories. Shigella toxin was isolated from S. dysenteriae 1 strain 60 R and purified as described previously (5) by affinity chromatography on Blue Sepharose followed by chromatofocusing and molecular sieving on Biogel P-60. The toxin subunits were separated by a modification of previously published procedures (Ref. 5 and A. Donohue-Rolfe, personal communication). The toxin was dissolved in 0.1 M glycine, pH 3.2 containing 8 M urea, incubated for 24 h at room temperature and applied to a Bio-Gel P-60 column. Fractions of 1 ml were eluted with the same buffer at a flow rate of 5 ml/h. Fractions containing the A and B subunits were identified by SDS polyacrylamide gel electrophoresis and pooled. Renaturation of the subunits was accomplished by slow dialysis against the elution buffer which was gradually exchanged over 20 h with 0.05 M Tris-HCl, pH 7.5. The protein was finally dialysed against 0.02 M ammonium bicarbonate.

Phosphatidylcholine or phosphatidylcholine/globotriaosyl ceramide vesicles were obtained by the sonication method. For the latter, 5 mg of DMPC in chloroform was mixed with 0.5 mg of glycolipid. The solvent was evaporated under nitrogen and the lipid film was dispersed in 1 ml of buffer (20 mM Hepes, 100 mM NaCl, pH 7.2), followed by sonication to optical clarity (approximately 15 min) with a probe type Branson sonifier. During the sonication, the temperature was maintained at approx. 50°C. Residual multilamellar liposomes were removed by centrifugation at 40 000 × g for 30 min.

Fluorescence experiments were performed with a SLM-800 spectrofluorimeter equipped with a thermostatic cell holder. Measurements were made on 200 µl samples in 3 mm path-length quartz cuvettes. The excitation wavelength was 290 nm. Fluorescence intensities were corrected for blank measurements from suspensions of lipid in buffer and for dilution effect. The emission maxima were determined from corrected spectra.

RESULTS

B-subunit. Previous studies have shown that the B-subunit of Shigella toxin, if properly renaturated after the isolation procedure, retains its specific physiological functions. Particularly, the isolated B-chains bind to the receptor sites on HeLa cells[1] and to rabbitt intestinal microvillus

[1]Donohue-Rolfe, A., Keusch, G.T., Auclair, F., Jacewicz, M., Seidah, N.G.; Abstract: Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans 1986.

membranes (12). This binding of B-subunit is specific and saturable as is the binding of holotoxin (12). Since each B-chain contains a single tryptophan residue (6), the interaction of these chains with the membrane receptor sites may be conveniently probed by fluorescence spectroscopy.

The emission spectrum of the B-subunit in aqueous solution (pH 7.2) has a maximum at 350 nm, indicating a very polar environment of the indole chromophore (15). The spectrum remains unchanged in the presence of receptor-free DMPC membranes (Fig. 1). Globotriaosyl ceramide-containing vesicles, on the other hand, induce a concentration- dependent shift of the fluorescence maximum, λ_{max}, to shorter wavelength until a plateau at 344 nm is reached (Fig. 1A). This shift in λ_{max} is accompanied by a marked increase in fluorescence intensity (Fig. 1B). At the threshold receptor concentration sufficient to shift λ_{max} to 344 nm, the relative emission intensity is enhanced by a factor of 2.1. Somewhat surprisingly, however, the intensity does not saturate at this level; it shows further enhancement with increasing concentration of the vesicles.

Additional information regarding the effect of receptor binding on the microenvironment of the single tryptophan residue in the B-subunit was obtained from fluorescence quenching experiments. The efficiency of quenching by an uncharged polar solute, acrylamide, provides a very sensitive measure of the exposure (or depth of "burial") of tryptophans in proteins (16,17).

Quenching of tryptophan fluorescence in the free B-subunit is very effective; the emission intensity is reduced by 50% at acrylamide concentrations as low as 70 mM (Fig. 2). Moreover, the upward curvature of the Stern-Volmer plot (Fig. 2) suggests the accessibility of tryptophan to both collisional and static quenching (16,17). Binding of B-subunits to the receptor results in a pronounced decrease in the quenching efficiency.

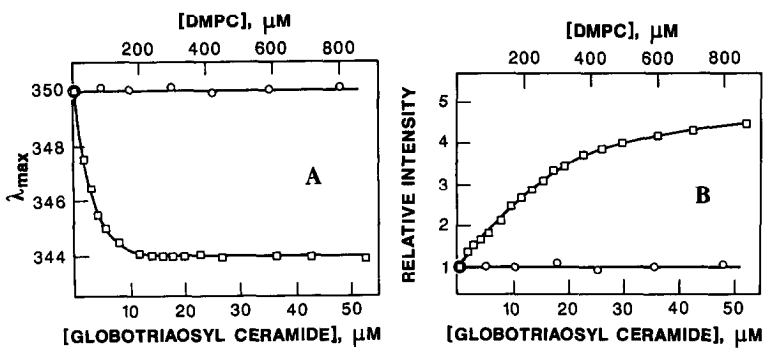
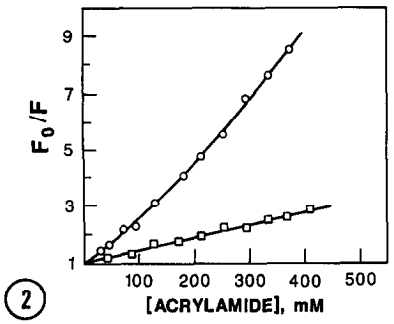


Fig. 1. Effect of lipid vesicles on the fluorescence of the isolated B-chain of Shigella toxin. (A): Maximum of fluorescence emission, λ_{max}. (B): Fluorescence intensity. Circles and upper scale represent DMPC vesicles alone; squares and lower scale represent DMPC vesicles containing 6 mol% of the globotriaosyl ceramide receptor. The concentration of Shigella toxin B-chain was 60 μg/ml (8 μM).



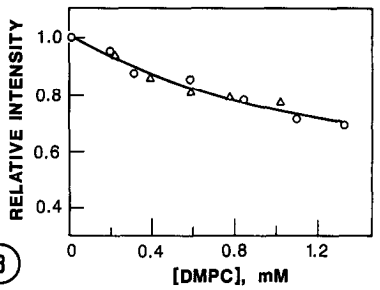


Fig. 2. Quenching of the fluorescence of Shigella toxin B-chain (8 μM) by acrylamide. Protein alone (O) and Protein in the presence of DMPC/globotriaosyl ceramide (94:6) vesicles (□). The concentration of globotriaosyl ceramide receptor was 40 μM.

Fig. 3. Effect of lipid vesicles on the fluorescence of Shigella toxin A-subunit. DMPC alone (Δ) and DMPC vesicles containing 6 mol% globotriaosyl ceramide (O). Protein concentration was 50 μg/ml.

Furthermore, the F_0/F vs. quencher concentration plot becomes linear, suggesting elimination of the static component of the quenching.

A rigorous analysis of the Stern-Volmer plots in terms of quenching rate constants is at present hampered by the lack of fluorescence lifetime measurements. Nonetheless, the existing data alone (Fig. 2) provide clear indication that upon binding of the B-subunit to the receptor, the unique tryptophan residue becomes considerably less accessible to the aqueous quencher. Alternatively, the reduced slope of the F_0/F vs. [acrylamide] plot could be due to a shorter fluorescence lifetime τ_0 (16,17). The latter, however, is unlikely in view of the increased fluorescence quantum yield in the receptor-associated protein (Fig. 1B).

A-subunit. The emission maximum in the fluorescence spectrum of the isolated A-subunit is at 332 nm, indicating that the average environment of tryptophan residues is much less polar as compared to that of a single tryptophan in the B-chain. In the presence of either globotriaosyl ceramide-containing vesicles or receptor-free DMPC vesicles, there is no change in λ_{max}. However, both types of vesicles produce a concentration-dependent decrease in fluorescence intensity (Fig. 3). Clearly, the latter effect is not specific to the globotriaosyl ceramide receptor, but rather reflects the ability of the A-subunit to interact directly with the bilayer membranes of phosphatidylcholine.

DISCUSSION

Both the position of the maximum in the emission spectrum and the efficiency of fluorescence quenching by acrylamide, indicate a very high degree of exposure of the single tryptophan residue in the B-subunit (Trp-34) to water. Notably, the quencher accessibility of the indole ring in the B-subunit is comparable to that observed for exposed chromophores in largely random

coiled peptides such as adrenocorticotropin or glucagon (16). The secondary and tertiary structure of the B-subunit have not been determined; however, the Chou-Fasman algorithms predict a highly ordered structure, with a large proportion of amino acids in α-helices and β-sheet (6). The tryptophan residue is thus most likely located at, or near the surface of the folded B-chain.

The main focus of this work was the interaction between the B-subunit of Shigella toxin and the membrane-associated receptor, globotriaosyl ceramide. Binding to the receptor produces perturbation in the immediate environment of the tryptophan residue. The indole ring becomes less accessible to the aqueous quencher (Fig. 2) and senses a more hydrophobic environment (Fig. 1A). This increase in hydrophobicity - although easily detectable by fluorescence spectroscopy - is, however, small; the position of λ_{max} in the emission spectrum of the receptor-bound B-chain is indicative of a still relatively polar environment. The changes in fluorescence properties most likely reflect a conformational change in the B-subunit, although the reduced exposure to the aqueous quencher may also be caused by the direct steric shielding of the indole ring by the oligosaccharide moiety of the receptor. In any case, the ability of Trp-34 to sense the interaction with globotriosyl ceramide suggests that this residue may be in or very near the receptor binding domain of the B-subunit. A similar "strategic" location of tryptophan has been suggested previously for the B-chain of cholera toxin (18,19).

The maximum shift in λ_{max} is reached at a molar ratio of total membrane-embedded glycolipid to B-chain of 1.4:1 (Fig. 1A). A further increase in the receptor concentration does not affect the position of the maximum in the emission spectrum, indicating that all protein present is already bound to the receptor. Thus, assuming that (i) globotriaosyl ceramide is uniformly distributed between the outer and inner monolayers of the sonicated DMPC vesicles and (ii) only approximately two thirds of the total glycolipid (that is, only the glycolipid associated with the outer monolayer (20)) is accessible to the protein, it can be estimated from the λ_{max} vs. receptor concentration plot that the stoichiometry of the isolated B-chain binding to globotriaosyl ceramide is 1:1.[2] Whether the ability of each B-chain to interact with one

[2]The observation that the fluorescence intensity does not saturate at the level corresponding to the equimolar concentrations of B-chain and accessible receptor - although not fully understood - by no means contradicts the above estimate. The secondary increase in fluorescence intensity (not accompanied by a further change in λ_{max}) is unlikely to represent the interaction of already bound B-chain with a second receptor molecule. The more plausible explanation is that the relatively high density of receptor sites in our model system leads to a relatively tight packing of B-chains on the membrane surface which results in a partial quenching of the tryptophan fluorescence. Upon addition of excess vesicles, there is a redistribution of B-subunits to new receptor sites; the resulting increase in the separation between protein molecules may lead to the elimination of fluorescence quenching.

molecule of globotriaosyl ceramide is preserved in the intact toxin remains to be determined. If so, this would indicate the possibility of multivalent binding of Shigella toxin to up to five receptor sites on the membrane surface.

Shigella toxin belongs to a family of bacterial toxins that are composed of two types of polypetide chains: the B-subunit which binds to the cell surface receptor and the toxic A-subunit which penetrates into the cytoplasm and exerts a biological effect (7,8). The central question related to the mode of action of these A-B type toxins is the mechanism by which the active A-chain traverses the hydrophobic barrier of a cell membrane. The system studied most extensively in this respect is cholera toxin, for which two mechanisms of membrane translocation have been proposed. The first model assumes that the B-subunits penetrate the lipid bilayer and form hydrophilic channels through which the A-chain is transported (21). The second proposal - supported by hydrophobic photolabelling (22,23), light scattering (24), and electron diffraction (25) experiments - is that the A-subunit traverses the membrane alone by direct interaction with the bilayer interior. In respect to Shigella toxin, our data are not in favour of the transmembrane channel model. There is no indication of an extensive penetration of B-chains into the lipid bilayer. Such a penetration would likely result in a nonpolar environment for Trp-34, particularly in view of the close proximity of this residue to a single hydrophobic segment of the B-chain (6). The fluorescence emission spectrum of the bound B-subunit, with a maximum at 344 nm, is far more red-shifted than would be expected for the membrane-embedded protein. It is rather more consistent with the view that the receptor-associated B-chain remains largely on the membrane surface.

The notion that the A-subunit traverses the membrane by routes other than putative B-subunit channels is fully supported by the observation that isolated A-chains can interact directly with receptor-free lipid bilayers (Fig. 3). In the whole toxin, this interaction - whose detailed nature is under current investigation - may be further facilitated by the pentameric B-subunit. Indeed, the initial receptor binding of B-chains automatically places the A-subunit within close proximity of the cell membrane; it may also produce a local destabilization of the bilayer, thus providing optimal conditions for the passage of the A-subunit through the hydrophobic membrane interior.

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