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Fluorescence Analysis of Galactose, Lactose, and Fucose Interaction with the Cholera Toxin B Subunit

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The cholera toxin B subunit (CTB) recognizes ganglioside GM1 receptors on target cells to facilitate entry of the toxin's A1 polypeptide into the host cytoplasm. GM1 binding to the CTB homopentamer occurs cooperatively with the most prominent interactions involving the terminal galactose residue of the ganglioside. Here, it is shown that association of galactose, lactose, or fucose (6-deoxy-galactose) with CTB is readily monitored using fluorescence spectroscopy. In many respects, however, the formation of CTB complexes with these small sugar analogues of GM1 greatly differs from the formation of complexes with the ganglioside itself. Each of these monosaccharides has a much weaker affinity for CTB than does GM1 and none of the sugars appear to be bound cooperatively. Moreover, GM1 binding conveys a stabilizing effect to CTB which is not seen upon binding of galactose or lactose. These data indicate that CTB-GM1 interactions involving sites other than the terminal galactose of the ganglioside serve prominently in the proper placement of CT on the target cell surface.

The major biochemical events elicited by the *Vibrio cholerae* enterotoxin (CT) are known (1, 2), but details of the cholera toxin B subunit (CTB) role in intoxication remain unclear. CTB binds to ganglioside GM1 to potentiate transport of the A1 polypeptide (CTA1) into the target cell cytoplasm where it exerts cytopathic effects. Improved understanding of this CTB-mediated protein movement will be useful for developing prophylactic strategies for cholera intoxication, understanding principles of peptide movement across membranes, and devising methods for the directed transport of proteins into eukaryotic cells.

CTB is a homopentamer with a hydrophilic pore in which polypeptide A2 (CTA2) resides. CTA2 is linked by a disulfide bridge to CTA1 (2) which is oriented away from the cell surface following CT binding to GM1 (3). How CTA1 migrates from this position to occupy a site within the cell cytoplasm is not known, but at no point is CTB insertion into the membrane involved (4, 5). Recent data indicate that endosomal processing of CT is needed for CTA1 transport (6, 7), suggesting that the toxin transiently resides in an endocytic vesicle from which CTA1 transport occurs concomitant with reduction of its disulfide bridge with CTA2 (7).

Published crystal structures of CTB complexed with the GM1 oligosaccharide and galactose have helped in elucidating important features of the GM1 binding site (8, 9). Here, fluorescence spectroscopy is used to contrast some of the important features of CTB's association with GM1 with the same features following CTB binding of small sugar analogues of GM1. The data presented here show that galactose, lactose and fucose bind to CTB and influence the fluorescence of its single tryptophan (W88) much like GM1 does, however, certain features of the binding of these sugars appear to significantly differ from those of GM1.

MATERIALS AND METHODS

3-(4′-Isothiocyantophenyl)-7-diethylamino-4-methylcoumarin (CPI) was from Molecular Probes, Inc. (Eugene, OR). CTB was from List Biological Laboratories, Inc. (Campbell, CA). Ganglioside GM1, fluorescein isothiocyanate (FITC),

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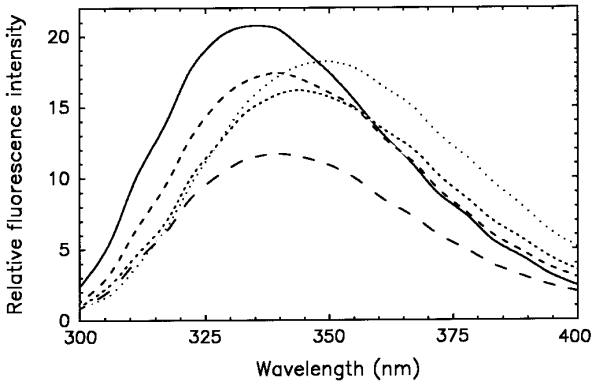

FIG. 1. Effect of ganglioside GM1 and sugar binding on the fluorescence emission spectrum of CTB. The fluorescence emission spectrum of W88 (0.5 μM CTB) was determined without any addition (dotted line), or with 2 μM GM1 (solid line), 200 mM galactose (long dashed line), 200 mM lactose (medium dashed line), or 250 mM fucose (short dashed line). Measurements were taken at 23°C in a final volume of 0.6 ml. Samples also contained 50 mM Tris-Cl (pH 7.5) and 0.15 M NaCl.

lactose, D-galactose, D-glucose, and D-fucose, were from Sigma Chemical Co. (St. Louis, MO). Other chemicals were reagent grade. CTB was resuspended to 1 mg/ml and used directly or passed over Sephadex G25 equilibrated with 0.1 M carbonate buffer (pH 8.5) for labeling with CPI or FITC as described previously (10).

Steady-state fluorescence measurements were taken on a Spex FluoroMax spectro-fluorometer (JY/Spex, Inc., Edison, NJ) equipped with excitation and emission polarizers and automatic correction for wavelength dependence of lamp intensity. Fluorescence from the tryptophan (W88) of each CTB monomer was measured with an excitation of 282 nm and an emission from 300 to 400 nm.

Overlap of W88 fluorescence emission with CPI absorption allows transfer of W88 excitation energy to CPI with an efficiency that is a function of their relative dipole orientations and the distance separating them (11). Fluorescence resonance energy transfer (FRET) efficiency and the distance between donor (W88) and acceptor (CPI) are related inversely to the sixth power, making FRET a sensitive method for observing distance changes within CTB. FRET efficiency (E) is determined from $E = 1 - (F_{DA} / F_D)$ [Equation 1] where F_{DA} is the W88 fluorescence intensity for CPI-CTB and F_D is the W88 fluorescence intensity for unlabeled CTB.

After determining E, the distance separating donor and acceptor can be calculated from $E = (R_O^6) / (R_O^6 + r^6)$ [Equation 2], where R_O is the distance giving 50% E and r is the observed distance between donor and acceptor (11). R_O is determined from the overlap of donor emission and acceptor absorption (11) assuming a random orientation of donor and acceptor dipoles. The latter assumption can be a source of error in these calculations (12) and an estimate of the possible error is included in the legend of Figure 3. FRET from W88 to CPI was measured with an excitation of 282 nm and emission from 300 to 500 nm.

RESULTS

GM1 binding to CTB induces a 12-nm bathochromic shift in the emission spectrum of W88 (13) (Figure 1) due to hydrophobic contacts involving the ganglioside's terminal galactose (8). A similar shift occurs when CTB binds galactose or lactose and a smaller shift is seen with fucose (6-deoxy-galactose) binding (Figure 1). No such effect was seen with glucose. These data show that W88 fluorescence is useful for monitoring CTB interactions with small sugars that mimic the terminal galactose of GM1. Interestingly, the shift in W88 emission caused by GM1 binding is accompanied by a slight increase in fluorescence intensity. In contrast, lactose and fucose binding cause a small decrease in fluorescence while galactose causes a large decrease in fluorescence (Figure 1). These observations suggest that galactose binding within the GM1 binding site may differ somewhat from that of the ganglioside.

To further analyze the binding of small sugars relative to that of GM1, CTB was titrated with each and concentration-dependent changes in W88 emission maximum monitored. As previously reported for GM1 and the GM1 oligosaccharide (14), CTB binding of GM1 appears

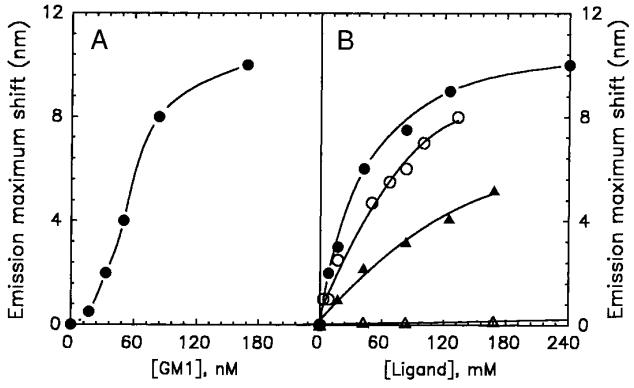

FIG. 2. The effects of ligand binding on the fluorescence emission maximum of W88. In **A,** CTB (0.1 μM) was titrated with nanomolar amounts of GM1 which are shown plotted against the magnitude of observed bathochromic shifts in the emission maximum of W88. In **B,** the same experiment was done using 0.5 μM CTB and millimolar concentrations of galactose (closed circles), lactose (open circles), fucose (closed triangles), or glucose (open triangles). Values shown are an average of triplicates with standard deviations not exceeding 10%.

to occur cooperatively (Figure 2A). In contrast, binding of galactose, lactose and fucose does not appear to be cooperative (Figure 2B). Differences in the binding of these sugars and GM1 also is apparent from the dissociation constants of each. Based on emission spectrum shifts, CTB has a dissociation constant (k_d) for GM1 of about 50 nM which is similar to previously reported values (2). In contrast, CTB has a k_d of about 40 mM for galactose, 81 mM for lactose, and over 150 mM for fucose. These large (10^6-fold) k_d differences indicate that interactions other than those involving the terminal galactose contribute significantly to the tight binding of GM1. Moreover, the absence of cooperative binding for these sugars implies that interactions between CTB and portions of GM1 other than the terminal galactose elicit communication between CTB monomers (most likely as a result of subtle changes in CTB structure).

To monitor the effects of ligand binding on its structure, CTB was labeled with CPI and the distance between this extrinsic probe and W88 was monitored by FRET in the absence and presence of galactose, lactose and GM1. In the absence of ligand, low pH causes a decrease in the apparent distance between the sites, presumably due to protein denaturation (Figure 3A). In contrast, CTB conformation is stabilized against low pH after association with GM1 (Figure 3A). When the same experiment was carried out using high concentrations of galactose or lactose, no ligand-induced stabilization was observed at low pH (Figure 3B). Identical results were seen using FITC-labeled CTB (data not shown).

DISCUSSION

In this study, fluorescence spectroscopy was used to examine the association of small sugar analogues representing the terminal galactose ''finger'' of GM1 with CTB. Like GM1, these sugars (including galactose, lactose and fucose) bind CTB to induce bathochromic shifts in the emission spectrum of W88; however, their binding is much weaker than that of GM1 and appears to lack the CTB structural stabilization conferred by GM1 binding (14).

Based on crystal structure analyses, the major contacts between CTB and GM1 involve the terminal galactose of the ganglioside (8, 9). Hydrophobic contacts involving this galactose residue are responsible for the 12-nm bathochromic shift observed in W88 emission upon CTB-GM1 association (9). GM1 binding occurs at the interface between adjacent CTB monomers (8) with most protein-sugar contacts involving one of the two monomers. Contacts with the

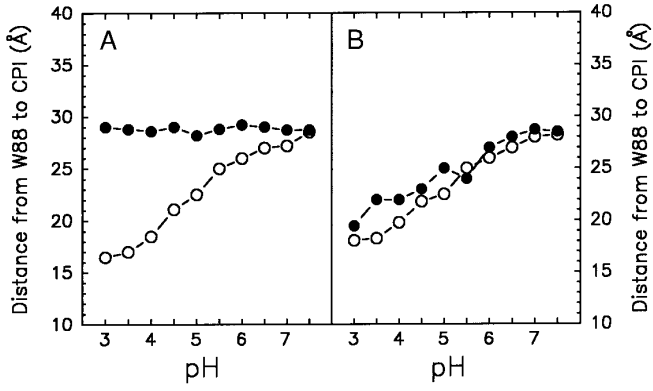

FIG. 3. The effect of GM1 and GM1-analogue binding on the stability of CTB at low pH. FRET was used to monitor the apparent distance from W88 to CPI. In **A,** open symbols are the observed distance separating donor and acceptor from pH 7.5 to 3.0 (in 0.5 pH increments) in the absence of GM1. The closed symbols show the same determinations in the presence of excess GM1. In **B,** the open symbols are the same determinations in the presence of 200 mM galactose and the closed symbols are in the presence of 200 mM lactose. Errors in these determinations were estimated by determining the polarization values for W88 and CPI. These values were used to approximate the degree of randomization of the donor and acceptor dipoles over the lifetime of the donor fluorescence (12). Half-height limits of uncertainty did not exceed 11% for any of the distance determinations shown.

adjacent monomer involve the C6 hydroxyl group of galactose (8, 9) and, from the data presented here (with galactose and lactose), it appears that these contacts are not responsible for the cooperative binding of GM1 and are not solely responsible for ligand-induced CTB stabilization. Moreover, demonstration that fucose is capable of binding CTB (Figures 1 and 2B) indicates that protein contacts with the C6 hydroxyl group of galactose are not essential for sugar binding, but are needed for optimal binding. This is supported by the observation that fucosyl-GM1 is capable of serving as a receptor for CT (15). Interestingly, fucose binding to CTB is not accompanied by the large decrease in W88 fluorescence intensity that accompanies galactose binding. This suggests that C6 hydroxyl interactions are important for precise positioning of galactose in the GM1 binding pocket of CTB.

Because galactose and lactose do not bind cooperatively to CTB, GM1 contacts other than those of the adjacent CTB monomer must be responsible for mediating the interprotein communication that facilitates cooperative GM1 binding. It seems likely that these contacts also help stabilize CTB structure against low pH. Lactose or galactose binding induces an ordering of CTB residues 51 to 60 (5, 9) which suggests that these sugars do elicit structural changes in CTB (perhaps mediated by direct contact between these regions and galactose). Moreover, lactose binding to LT causes the five C terminal amino acids of LTA2 to adopt a rigid α-helical structure (5), indicating that lactose does elicit interprotein structural changes within CT.

The role of CTB in the entry of CTA1 remains unclear; however, the fact that CTB is needed for CTA1 to elicit cytopathic effects on cells, even in tissue culture, would argue that this protein is more than a passive participant in the intoxication process. Moreover, it is possible that GM1-mediated stabilization of CTB at low pH may have an important role in the endosomal processing of CT. Recent findings by Lencer and coworkers (16) have provided evidence that CT is transcytosed from the apical to the basolateral face of a polarized intestinal epithelial cell line (16). These findings imply that CT spends a significant amount of time in endosomes where the pH can drop significantly. Therefore, when considering the mechanism of CT intoxication of enterocytes, it may be important to also consider the effects of low pH

on the protein's structure. Examination of the binding properties of GM1 analogues using fluorescence spectroscopy and analysis of the effects of GM1 analogues on CTB structure will be useful in this process.

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