Local Conformational Change in the B-Subunit of Shiga-like Toxin 1 at Endosomal pH†

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ABSTRACT: Shiga and Shiga-like toxins are potent bacterial cytotoxins composed of six proteins: one A-subunit that possesses a toxic *N*-glycosidase activity and a pentamer of identical B-subunits that anchors the toxin to glycolipids present on mammalian cells. Following their endocytosis through coated pits, a segment of the A-subunit noncovalently associated with the B oligomer is translocated to the cytoplasm where it enzymatically inactivates the protein synthesis machinery. The fluorescence intensity of the single tryptophan residue in the B-subunit is perturbed by pH conditions typically observed in an endosomal compartment, with a sharp reversible transition in fluorescence intensity occurring at pH 4.5. The secondary structure of the pentamer as monitored by circular dichroism is altered by pH conditions lower than 4.5 and greater than 10. However, the conformational change observed under acidic conditions as low as pH 2 does not parallel a loss of receptor binding potential and is reversible, suggesting that the structure of the B-subunit undergoes a second conformational change between pH 4.5 and 2 without a loss of tertiary or quaternary structure. The B-subunit may thus play a role in the translocation of the A chain to the cytoplasm, an event potentially mediated by a conformational change in its structure at pH levels occurring in the endosomal or lysosomal compartments.

Understanding the mechanism of entry of bacterial toxins into cells is of fundamental importance in the development of prophylactic approaches and of strategies to deliver drugs, antigens, and bioactive agents to cellular compartments. A particularly successful toxin design is the "*two-subunit assembly*" where one subunit (A-subunit) is an enzyme that specifically modifies a regulatory or essential component of the host cellular machinery while one or more copies of another subunit (B-subunit) is (are) involved in the recognition and binding of the toxin to surface receptors located on target cells. Examples of this structural design include diphtheria toxin, cholera toxin, the *Escherichia coli* heat-labile enterotoxins, and the Shiga (ShT)[1] and Shiga-like (SLT) toxins. The B-subunits of Shiga and related toxins (Seidah et al., 1986; Calderwood et al., 1987; DeGrandis et al., 1987; Jackson et al., 1987a,b; Strockbine et al., 1988) represent the smallest subunits (~70 amino acids) known to participate in this type of structural motif. These B-subunits spontaneously assemble into pentamers in solution (Ramotar et al., 1990; Head et al., 1991) and bind to unique cell-surface glycolipids (Lindberg et al., 1987; Lingwood et al., 1987). The oligomer noncovalently associates with a single A chain and is endocytosed through coated pits (Sandvig et al., 1989). The A chain must traverse the endosomal or lysosomal compartments and reach the cytoplasm for the toxin to be effective. In this report, results are presented which suggest that in addition to the three functions already carried out by this small protein (pentamerization, A-subunit, and glycolipid binding properties), the B-subunit may play a role in the mechanism of translocation of the A chain.

EXPERIMENTAL PROCEDURES

Materials. The glycolipid globotriaosylceramide (G_{b3}; Galα1–4Galβ1–4Glcβ1–1Cer) was purchased from Matreya, Inc. (Pleasant Gap, PA). The B-subunit of Shiga-like toxin 1 was overexpressed in *E. coli* (PJLB122) and purified as described previously (Ramotar et al., 1990). The homogeneity of the B-subunit preparation was confirmed by SDS–PAGE (single band) and by high-performance gel filtration chromatography (single peak eluting from Protein Pak 125 columns; Waters Associates, Millipore, Mississauga, Ontario).

Fluorescence Spectroscopy. Fluorescence spectra were recorded on a Perkin Elmer LS-3 fluorescence spectrometer (cell path length, 1.0 cm). The B-subunit concentration was 400 μg/mL dissolved in 0.15 M NaCl. Titrations were performed by adding known volumes of dilute HCl and NaOH solutions to a cuvette containing the protein sample. The measurements were obtained at 25 °C. The excitation wavelength was set at 298 nm with the maximal emission signal occurring at 350 nm. Fluorescence emission spectra were recorded from 320 to 410 nm.

Circular Dichroism Experiments. Circular dichroism spectra were recorded at 23 °C on a JASCO spectropolarimeter Model J-720 (cell path length, 0.1 cm). The B-subunit concentration was 140–160 μg/mL dissolved in 0.15 M NaCl. Individual samples were prepared for each pH condition tested, and the protein concentration was spectroscopically determined prior to and after spectrum collection using the extinction coefficient value at 278 nm for the B-subunit of 9500 M^{-1} cm^{-1} (monomer; Boyd, 1992). The pH titration experiments were performed in triplicate. Each spectrum represents the average of 12 accumulated spectra. The B-subunit has an isoelectric point of 5.7 (Boyd, 1992). No aggregation was observed at pH levels near this value as confirmed by the

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[1] Abbreviations: G_{b3}, globotriaosylceramide (Galα1–4Galβ1–4Glcβ1–1Cer), glycolipid receptor recognized by the B-subunit of Shiga and Shiga-like toxin 1; ShT, Shiga toxin; SLT, Shiga-like toxin; SLT-1, first variant of Shiga-like toxin to have been identified, also known as verotoxin 1 or VT1; SLT-1 B, B-subunit of Shiga-like toxin 1.

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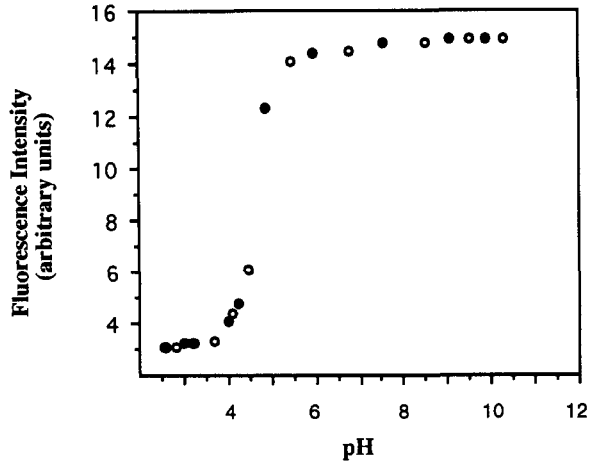

FIGURE 1: Effect of pH on the tryptophan fluorescence intensity of the B-subunit of Shiga-like toxin 1. Samples were initially titrated to low- and high-pH conditions (○) and then titrated back to neutral pH (●). λ_{exc} = 298 nm; λ_{em} = 350 nm.

recording of identical CD spectra for 10-fold-diluted samples of the B-subunit.

Receptor Binding Experiments. The B-subunit (30 μg in 20 μL of PBS, pH 7.2) was radioiodinated at room temperature for 12 min, by adding the protein sample to a polypropylene tube containing two Iodobeads (Pierce Chemical Co.) suspended in 200 μL of 100 mM sodium phosphate, pH 7.2, and 1 mCi of $Na^{125}I$. The radiolabeled protein was desalted on a Sephadex G-25 column (0.5 cm × 10 cm) preequilibrated with 10 mM sodium phosphate buffer, pH 7.2. The binding assay was performed following the method of Ramotar et al. (1990). Briefly, wells of flexible poly(vinyl chloride) 96-well plates were coated with 10 ng of G_{b3} (G_{b3}/PC/cholesterol, 2:10:5) in methanol/chloroform (9:1), and the solvents were allowed to evaporate overnight at room temperature. Wells were then blocked with 200 μL of 2% (w/v) BSA in PBS for 2 h at room temperature and washed 6 times with PBS (at the given pH) before ^{125}I-labeled B-subunit (3×10^4 cpm/well) was added either in the presence (50 μg; 1000-fold excess) or in the absence of unlabeled B-subunit. After incubation at room temperature for 2 h, the wells were washed 6 times with PBS (at the given pH) and cut, and the bound radioactivity was quantified in a γ-counter. To confirm that the denatured protein did not bind to its receptor in this assay, a sample of the B-subunit was boiled for 10 min and the assay subsequently performed at neutral pH. Error bars represent the standard deviation associated with experiments performed in triplicate.

RESULTS AND DISCUSSION

The objective of this study was to demonstrate the existence of one or more conformational states for the B-subunit of Shiga-like toxin 1 at acidic pH conditions that would suggest its involvement in the translocation mechanism of the A chain across the endosomal compartment. These states were contrasted with those arising from protein denaturation events based on the sensitivity of the receptor binding property to protein denaturation.

The Fluorescence of Tryptophan-34 Is Perturbed at Endosomal pH. The presence of a single tryptophan residue at position 34 in the sequence of the B-subunit allows us to monitor local pH-sensitive perturbations in the structure of the pentamer induced by pH changes using fluorescence spectroscopy. As shown in Figure 1, a sharp and reversible transition in fluorescence intensity occurs at pH 4.5. No shift in the wavelength maximum (350 nm) was observed under such conditions. From the crystal structure of the B-subunit pentamer, the tryptophan side chain in each monomer is located at the N-terminus of the single α-helix (residues 36–46; Figure 2A; Stein et al., 1992), forming a "doorway" to the central channel of the pentamer. The loss in fluorescence intensity below pH 5 could be interpreted as the result of an increased exposure of the indole ring to a polar environment. The crystal structure solved at neutral pH provides a clear indication that tryptophan-34 is exposed in all five subunits of the pentamer (Stein et al., 1992). Aspartic or glutamic acid side chains generally have pK_a values between 4.4 and 4.6 in the context of proteins (Cantor & Schimmel, 1980), suggesting that the ionization state of one or more of these side chains may influence the environment of Trp-34. Examination of the crystal structure of the pentamer does not place any such side chains in the vicinity of the indole ring. However, in three of the five subunits of the pentamer, the guanidinium group of arginine-33 is located less than 2.8 Å from side-chain oxygens of either Asp-16 or Asp-18 present on a loop region of an adjacent subunit (Figure 2B). This spacial proximity suggests the existence of a salt bridge that may be disrupted by the protonation of such aspartic acid side chains. The presence of a free guanidinium group at low pH may then influence the polarity of the tryptophan side-chain environment or destabilize the N-terminus of the α-helix through an unfavorable interaction with the helix dipole (Hol et al., 1978, 1981; Shoemaker et al., 1987).

The Structure of the B-Subunit Is Gradually Altered by pH Conditions Ranging from pH 2 to 4.5. The secondary structure of the B-subunit of Shiga-like toxin 1 (SLT-1 B) remains unaffected by pH conditions between 4.5 and 10 as identical spectra are recorded by circular dichroism in the far-UV region (195–250 nm). The spectrum of the B-subunit is altered as the pH of the sample drops below 5 (Figure 3A). These pH-sensitive spectral changes are reversible until pH 2 is reached. The pentamer is significantly denatured at pH conditions below 2 and above 10 (Figures 3B and 4) as the spectrum of the pentamer at neutral pH cannot be regenerated following the back-titration of such samples (results not shown). The circular dichroism spectra of SLT-1 B between pH 2 and 10 have negative maxima at 208 and 222 nm, supporting the presence of α-helices (Figure 3A; Brahms & Brahms, 1980). At neutral pH, residues 36–46 adopt an α-helical conformation which represents 16% of the secondary structure elements of the molecule (Stein et al., 1992). A graph of ellipticity values at 222 nm as a function of pH (Figure 4) suggests a change in α-helical content starting at pH conditions lower than 4.5, with ellipticity measurements at 222 nm increasing from −8100° at neutral pH to −9500° at pH 2 (Figure 4). The gradual change in conformation observed between pH 2 and 4.5 (Figures 3A and 4) is not concomitant with the sharp local perturbation observed for Trp-34 (Figure 1) and would suggest the existence of at least two conformational stages for the B-subunit pentamer at low pH. As discussed below, both states involve stable conformations rather than denatured states of the B-subunit since the pentamer retains its ability to bind the glycolipid G_{b3} under these conditions. The conformational transition occurring below pH 4.5 may be shifted to a higher pH (closer to endosomal pH, for example) in the context of the B-subunit being bound to a G_{b3}-containing membrane or in association with the A-subunit. Indeed, the pH-induced conformational change in the A chain of diphtheria toxin is sensitive to its environment, with the transition pH between hydrophilic and

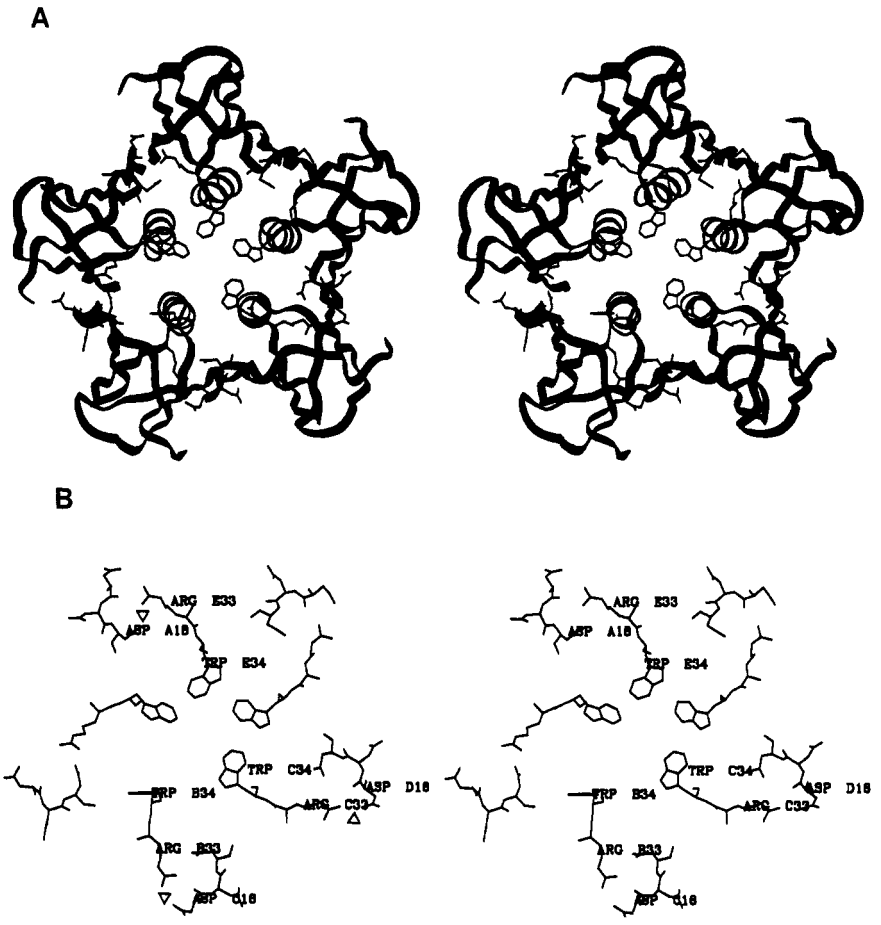

FIGURE 2: Stereoprojections of the B-subunit pentamer of Shiga-like toxin 1 [adapted from Stein et al. (1992)]. (A) Ribbonlike representation of the pentamer (Insight II software; Biosym Technologies, San Diego, CA). The single α-helix in each monomer gives rise to a close arrangement of five helices in the pentamer. The indole side chain of tryptophan-34 is located at the top of each helix. The B-subunits have identical sequences and are composed of 69 amino acids. Secondary structure composition of the B-subunit (Stein et al., 1992): β1, residues 3–8; β2, residues 9–14; β3, residues 20–24; β4, residues 27–31; α-helix, residues 36–46; β5, residues 49–53; β6, residues 65–68. The positions of Asp-16, Asp-17, Asp-18, and Arg 33 are illustrated in relation to Trp-34. (B) Identical representation as in (A) without the ribbons. Three possible salt bridges are highlighted (∇) where the distance separating the aspartic acid side-chain oxygens of either Asp-16 and Asp-18 and the guanidinium group of Arg-33 is less than 2.8 Å (see text). The subunits of the pentamer are alphabetically labeled from A to E, and the putative salt bridges would represent intersubunit contacts.

hydrophobic states being shifted from pH 3.5 to 5 in the presence of small unilamellar vesicles (Zhao & London, 1988). Its isolated B-subunit also undergoes a pH-induced exposure of a hydrophobic domain (Sandvig & Olsnes, 1981).

The Tertiary and Quaternary Structures of the B-Subunit Pentamer Are Stable between pH 2 and 10. The crystal structure of SLT-1 B-subunit has recently been derived from the X-ray diffraction analysis of crystals grown at neutral pH (pH 6.8–7.0; Stein et al., 1992). The protein exists as a pentamer. The peptide backbone adopts a structure reminiscent of the larger B-subunit of the *E. coli* heat-labile enterotoxin (Sixma et al., 1991, 1992; Stein et al., 1992). Each B-subunit is composed of six β-strands and one α-helix linked together by a series of short turns. Pentamerization occurs as a result of intersubunit contacts between two β-strands (β2 and β6) on adjacent B-subunits (Figure 2A; Stein et al., 1992). The SLT-1 B-subunit spontaneously assembles into pentamers in solution, with the presence of monomers or other multimers not being observed by gel filtration chromatography or by sedimentation equilibrium experiments (Ramotar et al., 1990). Monitoring the oligomeric state of the B-subunit by high-performance gel filtration chromatography at pH 5, thus approximating the pH of the endosomal or lysosomal compartments (Maxfield, 1982; Geisow, 1984; Geisow & Evans, 1984), revealed that the structure of the oligomer remains intact, eluting with the expected retention time and showing no sign of disaggregation (results not shown). A more important criteria proving the integrity of the B-subunit structure was to monitor the ability of the pentamer to bind to its natural receptor, the glycolipid globotriaosylceramide (G_{b3}) (Lingwood et al., 1987). Indeed, denaturation events, such as the reduction of the single disulfide bond in SLT-1 B followed by carboxamidomethylation, lead to the dissociation of the pentamer and result in a loss of the receptor binding property (Boyd, 1992). In this assay, G_{b3} was immobilized onto the solid phase, and radioiodinated SLT-1 B was allowed to associate with the bound receptor (Ramotar et al., 1990). The radiolabeled subunit was incubated in a range of pH conditions, prior to and during the binding step. The level of specific binding was assessed by performing the binding step in the presence and absence of an excess of unlabeled B-subunit. From the histogram presented in Figure 5, one can conclude that the specific binding of the toxin to its receptor is not affected by acidic pH as low as 3.5. A significant level of specific binding still occurs even after exposing SLT-1 B to pH levels below 2. A similar result was observed when the radiolabeled B-subunit was allowed to bind initially to G_{b3} at neutral pH and the complex was later exposed to lower pH conditions (pH 3 and 5; results not shown). The stability of the tertiary or quaternary structure was more sensitive to alkaline conditions, particularly pH levels above 10. Boiling the radiolabeled B-subunit for 10 min prior

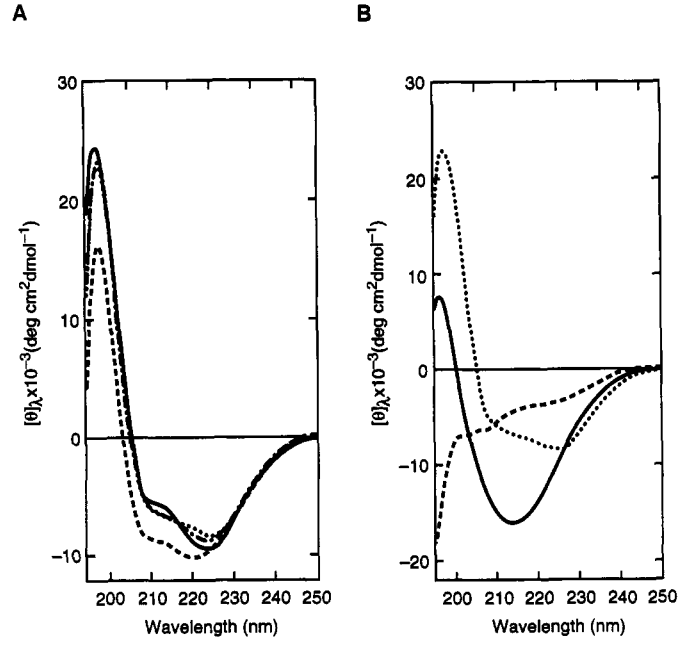

FIGURE 3: Effect of pH on the circular dichroism spectrum of the B-subunit of Shiga-like toxin 1 (195–250-nm region). (A) Spectra were recorded under neutral and acidic pH conditions: pH 7.0 (···), pH 4.0 (– · –), pH 3.0 (—), and pH 2.0 (- - -). (B) Spectra of denatured forms of the B-subunit at pH 1.3 (—) and pH 12.0 (- - -) are contrasted with the reference spectrum of the B-subunit recorded at pH 7.0 (···). Each spectrum represents the average of 12 accumulated spectra.

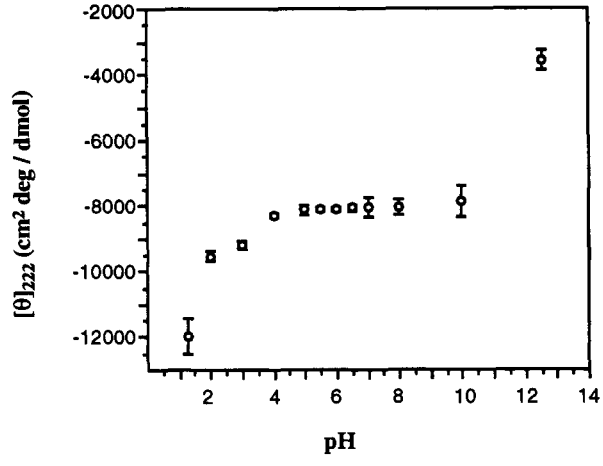

FIGURE 4: Effect of pH on ellipticity values at 222 nm. Ellipticity measurements ($[\theta]_{222mm}$) were derived from spectra recorded as described in Figure 3. The graph was constructed using spectra collected from three independent titrations. The pH and protein concentration of each sample were determined prior and after spectrum acquisition. The ellipticity data were extracted from individual spectrum representing the average of 12 accumulated spectra.

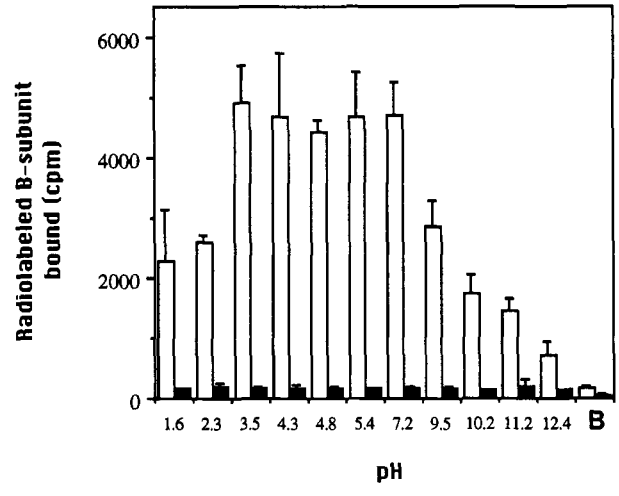

FIGURE 5: Histogram depicting the effect of pH on the specific binding of radioiodinated Shiga-like toxin 1 B-subunit to its natural receptor G_{b3}. Open bars, total binding; closed bars, nonspecific binding (presence of excess unlabeled B-subunit). B, denatured B-subunit as a result of boiling the protein sample prior to performing the binding assay. Error bars represent the standard deviation associated with experiments performed in triplicate.

to performing the binding assay resulted in a total loss of binding to G_{b3} (Figure 5). In summary, the changes observed in the tryptophan fluorescence and circular dichroism experiments under acidic pH conditions do not parallel a loss in tertiary or quaternary structure. These changes suggest the existence of discrete conformational states at low pH. A similar effect was not observed at alkaline conditions where the denaturation as monitored by loss of the receptor binding property correlates with large changes in the ellipticity values at 222 nm (Figure 4).

A Possible Role for the B-Subunit in A-Chain Translocation? In the structural context of the SLT-1 B-subunit pentamer, five α-helices line the central pore of a doughnut-shaped structure (Figure 2A; Stein et al., 1992). In the case of the crystal structure of the *E. coli* heat-labile enterotoxin, the C-terminal part of the A chain (A_2 fragment) is inserted into the central pore of the B-subunit pentamer, forming numerous contacts with a similar array of five helices (Sixma et al., 1991, 1992). One can postulate that the α-helices present in the SLT-1 B pentamer may represent the site of conformational change and of contact with the A_2 fragment of its A-subunit (Olsnes et al., 1981; O'Brein & LaVeck, 1983). The observation of a local conformational change in the B-subunit pentamer at acidic pH predicted for compartments associated with endocytosis suggests that the B-subunit may encode a mechanism for promoting or actuating the processing, release, and/or translocation of the A chain to the cytoplasm. The potential role of the B oligomer remains unclear but could involve the five α-helices. This site would be favored by analogy with the *E. coli* heat-labile enterotoxin where most of the A-chain contacts with its B-subunit pentamer occur through the central helices (Sixma et al., 1991, 1992).

Tryptophan fluorescence intensity (Figure 1) plateaus at pH levels above 5, implying a local change occurring near the NH_2 terminus of these helices. The crystal structure of the B-subunit indicates that the tryptophans in the pentamer are already mostly exposed to the solvent at neutral pH. Thus, at pH levels below 5, either the indole side chains must be located in proximity of charged side chains that are revealed within this pH range or the tryptophans and potentially the α-helices are partially reoriented to a more solvent-exposed environment. The small negative increase in negative ellipticity at 222 nm ($\sim$1400°) observed going from pH 7 to pH 2 also suggests an alteration in the position of α-helices or helical content. Finally, Gill (1976) suggested that the A_1 domain of cholera toxin may traverse the cytoplasmic membrane with the aid of the A_2 chain. In his proposed model, the central pore created by the association of five B-subunits acted as the transmembrane channel. Although the B-subunits of Shiga and related toxins are smaller ($\sim$25%) than those of cholera and *E. coli* heat-labile enterotoxins, the diameter of the central pore created in both pentamers is similar.

In support for the location of this conformational change, we have recently observed that the far-UV CD spectrum of the B-subunit remains unaffected by its complexation with a tetrasaccharide analogue of the receptor.[2] Since the B-subunit selectively binds the glycolipid G_{b3} and the receptor analogue, it suggests that the receptor binding domain of the B-subunit maintains a rigid tertiary or quaternary structure and that the receptor binding function is not influenced by acidic pH levels (Figure 5). The receptor binding domain has been postulated to reside in a cleft at the interface between β-strands of adjacent B-subunits (Stein et al., 1992), which would place the region of conformational change at a site removed from this receptor binding domain. Similarly, the maintenance of the pentameric state at pH 5 as monitored by gel filtration chromatography suggests that buried intra- and intersubunit contact sites between β-strands probably do not represent the segments undergoing a conformational change. Considering the small size of the B-subunit monomer, this systematic elimination of secondary structure elements potentially involved in the structural change observed at low pHs suggests the centrally-located α-helices as the site of conformational change. The SLT-1 B-subunit has recently been crystallized at pH 4.3, and crystals suitable for diffraction analysis should provide some insight into the nature of the conformational change observed.[3]

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[2] M. Saleh, B. Boyd, C. M. Kay, and J. Gariépy, unpublished observations.

[3] X. Lee, M. Saleh, and J. Gariépy, unpublished observations.

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