A pH-Dependent Conformational Change in the B-Subunit Pentamer of *Escherichia coli* Heat-Labile Enterotoxin: Structural Basis and Possible Functional Role for a Conserved Feature of the AB_5 Toxin Family†

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ABSTRACT: The non-covalently associated B-subunit moieties of AB_5 toxins, such as cholera toxin and related diarrheagenic enterotoxins, exhibit exceptional pH stability and remain pentameric at pH values as low as 2.0. Here, we investigate the structural basis of a pH-dependent conformational change which occurs within the B_5 structure of *Escherichia coli* heat-labile enterotoxin (EtxB) at around pH 5.0. The use of far-UV CD and fluorescence spectroscopy showed that EtxB pentamers undergo a fully reversible pH-dependent conformational change with a pK_a of 4.9 ± 0.1 (R^2 = 0.999) or 5.13 ± 0.01 (R^2 = 0.999), respectively. This renders the pentamer susceptible to SDS-mediated disassembly and decreases its thermal stability by 18 °C. A comparison of the pH-dependence of the structural change in $EtxB_5$, with that of a mutant containing a Ser substitution at His 57, revealed that the pK_a of the conformational change was shifted from ca. 5.1 to 4.4. This finding suggests that protonation of the imidazole side chain of His 57 might facilitate disruption of a spatially adjacent salt bridge, located between Glu 51 and Lys 91 in each B-subunit, thus triggering the conformational change in the pentameric structure. The pH-dependent conformational change was found to be inhibited when B-subunits bound to monosialoganglioside, G_{M1}; and to have no effect on the stability of interaction between A- and B-subunits within the AB_5 complex. This suggests that the conformational change is unlikely to have a direct involvement in toxicity. Conservation of the pH-dependent conformational change in the AB_5 toxin family, combined with the potential exposure of the hydrophobic core of β-barrel in the monomeric units, leads to the proposal that the conformational change may be the common feature that ensures the secretion of these proteins from the *Vibrionaceae*.

Heat-labile enterotoxins (Etx)[1] from enterotoxinogenic *Escherichia coli*, of human (hEtx) or porcine (pEtx) origin, are members of a family of hexameric AB_5 toxins which includes the highly homologous cholera toxin (Ctx) from *Vibrio cholerae*, shiga toxin from *Shigella dysenteriae*, and verotoxin I from *E. coli*. The A-subunits of each toxin either possess ADP-ribosyl transferase activity (Etx/Ctx; Cassel & Pfeuffer, 1978; Gill & Richardson, 1980) or cleave a specific adenine from 28S ribosomal RNA (verotoxin/shiga toxin; Endo *et al.*, 1988); while the B-subunits act as a carrier system mediating toxin internalization by binding to the ganglioside G_{M1} (Etx/Ctx; Cuatrecasas, 1973; Eidels *et al.*, 1983; Holmgren *et al.*, 1985) or the glycolipids G_{b3} and G_{b4} (verotoxin/shiga toxin; Jacewicz *et al.*, 1986; Lingwood *et al.*, 1987; Lindberg *et al.*, 1987; Tyrell *et al.*, 1992). Although the B-subunits of shiga toxin and verotoxin I show negligible sequence identity with those of Etx or Ctx, they each assemble into pentameric oligomers which share a common structural motif known as the oligosaccharide binding, or OB, fold (Murzin, 1993; Sixma *et al.*, 1991, 1992, 1993a,b; Stein *et al.*, 1992; Zhang *et al.*, 1995a,b). Though much work has been done on the biogenesis and toxicology of this class of toxins [for reviews see Spangler (1992), Hirst (1995), and O'Brien *et al.* (1992)], several key aspects remain unresolved. For example it has been speculated that, as for diphtheria toxin (Sandvig & Olsnes, 1980; Draper & Simon, 1980), a pH-dependent conformational change in the structure of the toxins may have some physiological relevance (Hirst, 1995; Hirst *et al.*, 1995; Saleh & Garuépy, 1993), but such changes are not well-defined.

pH-dependent conformational changes within the B-subunit pentamers of verotoxin I and Ctx have been previously reported. By monitoring changes in the intrinsic fluorescence of the protein, the B-subunit of verotoxin I was shown to undergo a conformational change with a mid-point of 4.5 (Saleh & Garuépy, 1993). Similar studies on the B-subunit of cholera toxin (CtxB) have reported that its intrinsic fluorescence decreases with pH, with an inflection at pH 5.8 (Bhakuni *et al.*, 1991) or associated with pK_a's of 4.4 and 6.2 (De Wolf *et al.*, 1985). The conformational change in CtxB has also been reported to result in increased sensitivity to trypsin at pH 5.0 (Bhakuni *et al.*, 1991); a decrease in thermal stability, with an inflection at pH 5.3,

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[1] Abbreviations: CtxB, B-subunit of cholera toxin; E_a, activation energy; EtxB, B-subunit of heat-labile enterotoxin; G_{M1}, monosialoganglioside G_{M1}; hEtx, heat-labile enterotoxin originating from enterotoxinogenic *E. coli* of human origin; pEtx, heat-labile enterotoxin originating from enterotoxinogenic *E. coli* of porcine origin; hEtxB, B-subunit of hEtx; pEtxB, B-subunit of pEtx.

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and an associated decrease in co-operativity of disassembly (Bhakuni *et al.*, 1991); and an increase in hydrophobicity by pH 4.2 (De Wolf *et al.*, 1987). In addition, the pH-dependent changes in the intrinsic fluorescence of CtxB were found to be abolished upon interaction of the B-subunit with its receptor, G_{M1} (De Wolf *et al.*, 1985).

The observation that a pH-dependent conformational change is conserved among the family of AB_5 toxins suggests that it may have a physiological significance. Indeed, the conformational change in verotoxin I has been ascribed a role in the toxicity of the protein (Saleh & Garuépy, 1993), on the basis that the protein was only likely to be in such a low pH environment after being internalized into endosomal compartments of the target cell. An alternative putative role suggested for the conformational change is in secretion from the pathogen, e.g., of Ctx from *V. cholerae* (Hirst *et al.*, 1995). A comprehensive characterization of the conformational change may allow for elucidation of the molecular mechanism by which the conformational change occurs. In turn, determination of the molecular basis may give an insight into the physiological significance of the conformational change.

In this paper we have characterized the pH-dependent conformational change that occurs in hEtxB pentamers, resulting from protonation of a group with a pK_a around 5.1. We show that this conformational change does not induce disassembly of the pentameric complex or modulate its ability to interact stably with its receptor, G_{M1}, or cause release of the A-subunit from the B pentamer. However, the structural change does result in a destabilization of the quaternary structure of hEtxB, both to elevated temperatures and to denaturants, in comparison with pentamers held at neutral pH. Our results suggest that the pH-dependent conformational change is unlikely to have a direct involvement in toxicity. The pK_a of the conformational change suggested it may be due to protonation of an imidazole side chain of histidine. Since the observed pH-dependent conformational change occurred in both hEtxB and in pEtxB (which have His residues at positions 13 and 57, and 57 only, respectively) we investigated if a mutant hEtxB-subunit containing a His 57 to Ser substitution showed a similar pH-dependent conformational change. This revealed that the conformational change in hEtxB is not abolished by the mutation; however, it was shifted from around pH 5.1 to pH 4.4, thus implicating a role for His 57 in modulating the pH-dependence of the structural change.

MATERIALS AND METHODS

Purification of Proteins. hEtxB was purified from the media of a rifampicin-resistant derivative of *Vibrio* sp. 60 (Leece & Hirst, 1992) expressing pMMB68 (Sandkvist *et al.*, 1987) by the method described previously (Amin & Hirst, 1994; Ruddock *et al.*, 1995). pEtxB, expressed from plasmid pMMB197 (Sandkvist, 1992), was purified by a modified version of the hEtxB purification protocol; hydrophobic interaction chromatography was performed at pH 8.9 using a phenyl Sepharose high substitution column (Pharmacia); ion exchange chromatography was performed at pH 7.5 using a Mono S cation exchange column. hEtx, expressed from plasmid pTRH29 (Yu *et al.*, 1992), was purified by a modified version of the hEtxB purification protocol; ammonium sulfate precipitation was performed at 40% w/v; ion exchange chromatography was performed at pH 8.75 using a Mono Q anion exchange column.

When the purified preparations of hEtxB and pEtxB were analyzed by SDS polyacrylamide gel electrophoresis and silver stained, only bands corresponding to the B-subunits were detected. When a purified preparation of hEtx was analyzed by SDS polyacrylamide gel electrophoresis under reducing conditions, bands corresponding to the A_1-, A_2-, and B-subunits were detected. This indicates that the purified hEtx was in a proteolytically nicked form (necessary for biological activity; Mekalanos *et al.*, 1979). The yield of purified protein was 10.8, 2.3, and 0.2 mg/L of *Vibrio* sp. 60 culture for hEtxB, pEtxB, and hEtx, respectively. Protein concentration was determined using the Bio-Rad protein microassay according to the instructions recommended by the manufacturer. The concentrations of hEtxB and pEtxB pentamers were determined using a G_{M1} ELISA as described previously (Amin & Hirst, 1994).

SDS Polyacrylamide Gel Electrophoresis. SDS polyacrylamide gel electrophoresis was performed on Bio-Rad Protean II or Mini Protean II systems, according to the conditions recommended by the manufacturer. Low molecular weight range molecular markers supplied by Bio-Rad were loaded in the outside lanes of each gel. Gels were stained with 0.05% (w/v) Coomassie blue R250 in 40% (v/v) methanol and 10% (v/v) glacial acetic acid.

Buffers and Solutions. The buffers used were McIlvaine buffer (0.2 M disodium hydrogen phosphate, 0.1 M citric acid; pH 7.5 to 2.5) All buffers were prepared fresh daily using constituents from Sigma/Aldrich and ELGA-purified water. All buffers for spectroscopic measurements were filtered through sterile 0.2 μm filters before use. The pH of buffers was determined using a Corning 240 pH meter calibrating at 4.00 and 7.00 with standard buffers supplied by Aldrich. pH calibration was checked using standard buffers of pH 2.00 and 3.00 supplied by BDH.

pH-Dependence of Disassembly and SDS Susceptibility of B Pentamers Determined by SDS Polyacrylamide Gel Electrophoresis. Samples of purified hEtxB (stock 3−6 mg/mL in PBS) were diluted to a concentration of 0.75 mg/mL in buffer ranging from pH 7.50 to 1.50 at 0.50 pH unit intervals, in the presence or absence of 1% w/v SDS. After 5 min at room temperature, 10 μL of each sample was added to 90 μL of McIlvaine buffer pH 7.0 and the samples briefly mixed. Twenty seconds after mixing, 25 μL of 5× SDS sample buffer was added to each sample and the samples briefly mixed. The samples were examined by SDS polyacrylamide gel electrophoresis.

pH-Dependence of Receptor Binding of B Pentamers Determined by G_{M1} ELISA. Samples of purified hEtxB (stock 6 mg/mL in PBS) were diluted to a concentration of 1 μg/mL in buffers ranging from pH 7.50 to 2.50. The concentration of receptor binding competent hEtxB pentamers was determined using a G_{M1} ELISA (Amin & Hirst, 1994).

pH-Dependence of A−B Interactions Determined by G_{M1} ELISA. (i) Interactions in Solution. Samples of purified hEtx (stock 100 μg/mL in PBS) were diluted to a concentration of 10 μg/mL in buffers ranging from pH 7.0 to 4.0. After 15 min at 25 °C the samples were diluted to 1 μg/mL in PBS and the concentration of A- and B-subunits determined using a G_{M1} ELISA. B-subunits were detected as described previously (Amin & Hirst, 1994); detection of A-subunit used a rabbit polyclonal αR833 at 1 in 5000 as

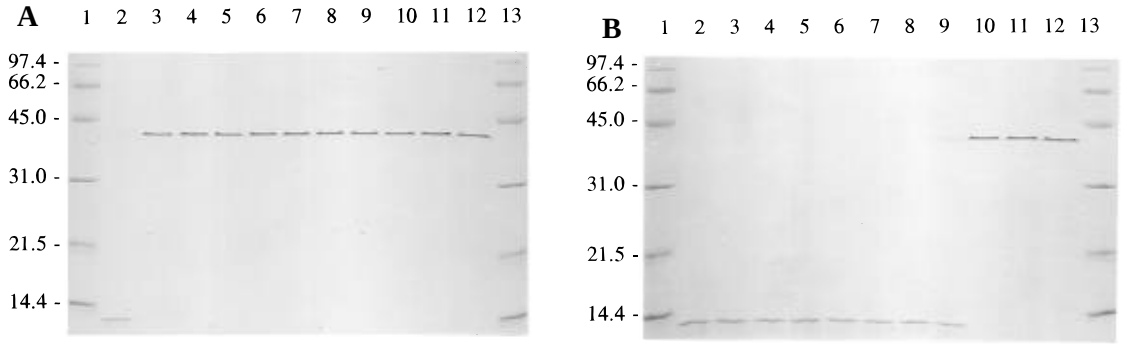

FIGURE 1: Acid-mediated disassembly and SDS susceptibility of hEtxB. (A) Equal amounts of hEtxB were applied to each lane of an SDS polyacrylamide gel after being subjected to buffers of differing pH and subsequent neutralization. Lanes 1 and 13, molecular weight markers; lanes 2−11, pH 1.5−6.0 in 0.5 pH unit incremental steps; lane 12, pH 7.0. Mass of markers, in kDa, indicated. (B) As in A, but hEtxB was subjected to buffers of differing pH in the presence of 1% w/v SDS, before neutralization.

the primary antibody and HRP-labeled goat anti rabbit at 1 in 5000 as the secondary antibody (Streatfield *et al.*, 1992).

(ii) Interactions after Binding to G_{M1}. Samples of purified hEtx were diluted to 1 μg/mL in PBS. After binding to G_{M1} immobilized on an ELISA plate these samples were incubated for 15 min in buffers ranging from pH 7.0 to 4.0 and then washed in PBS. The concentrations of A- and B-subunits were then determined by ELISA.

Spectroscopic Methods. Circular dichroism spectra were recorded on a Jasco J600 spectropolarimeter. A cell with a path length of 0.05 cm, a spectral band width of 1 nm, and a time constant of 2 s were used, and each spectrum was recorded as an average of 4 scans. Circular dichroism measurements were made at 25 °C on samples prepared by a 10× dilution of protein stock solution into 1 mL of buffer (pH 7.50−2.50); samples were mixed and allowed to reach equilibrium for 15 min at room temperature. Molar ellipticity values were calculated using a value of 113.6 for the mean residue weight.

Fluorescence measurements were made on a Perkin-Elmer LS50 spectrofluorimeter. Fluorescence measurements were made on samples prepared by a 50× dilution of protein stock solution into 1 mL of buffer (pH 7.50−2.50), in the presence or absence of a 40-fold molar excess over protein of G_{M1}. Emission spectra were averaged over three scans, after thermal equilibration to 25 °C, at an excitation wavelength of 280 nm, with 5 nm excitation and emission slit widths.

Mutagenesis of EtxB. The His 57 sequence was mutated in plasmid pTRH29, a derivative of the plasmid vector pBluescript II KS+, that contains the genes for the A and B subunits of *E. coli* heat-labile enterotoxin (Yu *et al.*, 1992). The mutagenesis was performed with an *in vitro* oligonucleotide-directed mutagenesis kit (Amersham International) using single-stranded pTRH29 as a template and a synthetic oligonucleotide (5′-GAGTCTATAGATTGACTGC-3′) from Cruachem Ltd as the mutagenic primer. The correct His to Ser substitution was confirmed by dideoxy sequencing using sequenase II (United States Biochemical Corp.), and the resultant plasmid was designated pTRH74. The mutant *etxB* gene from pTRH74 was excised by digestion with *Eco*RI and *Spe*I restriction enzymes and ligated into the same sites in pTRH56 (Nashar *et al.*, 1996) to yield a broad host range expression vector, pTRH75, expressing H57S hEtxB. After electroporation into *Vibrio* sp. 60, H57S hEtxB was purified from the media in an identical manner to that used for the purification of wild type hEtxB. The yield of purified protein was 6 mg/L of *Vibrio* sp. 60 culture.

Data Analysis. Kinetic data and regression lines were analyzed using Igor v1.21, WaveMetrics Ltd.

RESULTS AND DISCUSSION

Detection and Characterization of a pH-Dependent Conformational Change in the Structure of Pentameric EtxB

It has been reported (Bhakuni *et al.*, 1991) that a conformational change occurring in CtxB around pH 5 could be demonstrated by an increase in the susceptibility of the protein to digestion by the protease trypsin. Despite the 80% identity between EtxB and CtxB, including full conservation of arginine and lysine residues, hEtxB was shown not to become susceptible to trypsin over the pH range 7.0−4.0 (data not shown). However, evidence for a conformational change in the pentameric structure of EtxB around pH 5.0 was obtained from experiments showing that the pentamer became more susceptible to dissociation when this pH was combined with some other denaturing agent, namely the ionic detergent SDS or elevated temperature.

At neutral pH, the native EtxB pentamer is stable in the presence of 1% w/v SDS and so the pentamer runs undissociated in SDS polyacrylamide electrophoresis gels, at an apparent molecular weight of 42 kDa. Hence, if hEtxB is subjected to acid treatment over a range of pH down to 1.5, and is then neutralized and analysed by SDS−PAGE (Figure 1A), the products resolve as the intact pentameric species, except after treatment at the lowest pH where the monomeric species is detected, running at an apparent molecular weight of 12 kDa. This is consistent with previous work showing that pH below 2.0 is required in order to disassemble the pentamer (Ruddock *et al.*, 1995). If on the other hand, the acid treatment is carried out in the presence of SDS, before neutralization and SDS−PAGE analysis, then the products are disassembled, in every case where the acid plus SDS treatment is at pH 5.0 or below (Figure 1B). Since neither acid treatment (above pH 2.0) nor SDS treatment alone disassembled the pentamer, it appears that the moderate acid pH (pH 5.0 to 2.0) renders the pentamer sensitive to SDS-mediated disassembly.

Further evidence was generated by examining the pH-dependence of the thermal stability of the protein. This was determined by heating the protein for 5 min, at temperatures over the range 25−95 °C, allowing to cool and then examining the intrinsic fluorescence of the protein at pH 7.0 (EtxB does not reassemble from the thermally denatured state

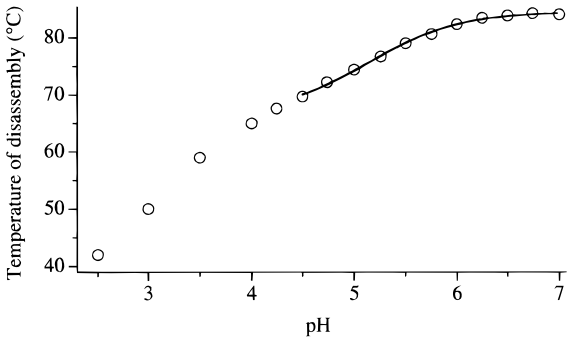

FIGURE 2: pH-dependence of the thermal stability of hEtxB at 0.52 mg/mL determined by fluorescence spectral differences between pentameric and monomeric species, at 21 μg/mL, pH 7.0. Line of best fit is to a single pK_a-dependent event (over the pH range 7.0 to 4.5).

at 25 °C).[2] Upon the transition from pentamer to monomer the intrinsic fluorescence of the protein undergoes a marked change, with a decrease in the emission intensity at 350 nm and the appearance of a distinct peak around 310 nm. This is consistent with a decrease in the non-radiative energy transfer from tyrosine residues in the protein to the single tryptophan residue upon loss of a compact quaternary/tertiary structure. The amount of thermal disassembly was determined by monitoring the ratio of emission at 350 nm to 310 nm with an excitation of 280 nm, since this method allows for concentration independent determination. At all pH values the transition from pentamer to monomer occurred over a few degrees centigrade. At pH 7.0 the mid point of thermal disassembly occurred at 84 °C. This is comparable with the value obtained by Fourier transform IR spectroscopy for unfolding of CtxB of 66 to 78 °C (Surewicz *et al.*, 1990). A plot of the midpoint of thermal mediated disassembly against pH showed that the thermal stability of the protein decreased with decreasing pH (Figure 2). Over the pH range 7.5−4.5 this decrease fitted a model based on a single pK_a-dependent event, with a pK_a of 5.14 ± 0.03 (R^2 = 0.998) and a decrease in thermal stability of 18 °C. Thermal stability below pH 4.5 showed a rapid decrease with decreasing pH. This decrease probably arises due to the loss of stabilising inter- and intra-subunit salt bridges within the protein, as the side chains of glutamic acid and aspartic acid residues are protonated.

The studies on disassembly of the EtxB pentamer at moderately acid pH suggest that a conformational change in the pentamer at around pH 5.0 is responsible for its susceptibility to thermal or detergent-induced disassembly. In order to elucidate more fully the nature of the conformational change occurring in EtxB, the pH-dependence of the spectroscopic properties of the protein were studied. EtxB contains a single tryptophan per monomer at residue 88. At pH 7.0, EtxB showed fluorescence properties characteristic of an exposed tryptophan group, with an emission maximum at 346 ± 1 nm when excited at 280 nm. Over the pH range 7.0−4.0 the fluorescence intensity of EtxB decreased with decreasing pH, with no change in λ_{max} (Figure 3A). This decrease fitted a model based on a single pK_a-dependent event, with a pK_a of 5.13 ± 0.01 (R^2 = 0.999) or 5.03 ± 0.02 (R^2 = 0.999) for hEtxB and pEtxB, respectively. Although intrinsic fluorescence allows for the direct determination of the pK_a of the affector, it only monitors changes in the local environment of the reporter groups, in this case tryptophan 88. To determine the pH-dependence of global changes in secondary structure, far-UV circular dichroism was used. Over the pH range 7.0−4.0 a pH-dependent change was observed in the far-UV CD spectrum of hEtxB (Figure 3B). Monitoring the change in the molar ellipticity at 218 nm with pH showed that the conformational change determined by CD fitted a model based on a single pK_a-dependent event, with a pK_a of 4.9 ± 0.1 (R^2 = 0.999; data not shown).

[2] L. W. Ruddock, S. P. Ruston, and T. R. Hirst, unpublished observations.

Analysis of the Role of Histidine 57

Spectrophotometric methods for examining both local and global changes in EtxB pentameric structure in moderately acidic conditions have shown that a single pK_a-dependent event occurs with a pK_a around 5.13. This may arise from the loss of a salt bridge, though this pK_a is higher than usually expected for glutamic or aspartic acid side chain carboxylates, or from protonation of a residue which results in a structure destabilizing interaction, e.g., protonation of a buried neutral side chain. Although slightly lower than that usually expected, the pK_a may represent the protonation of the imidazole side chain of histidine. hEtxB contains two histidine residues at positions 13 and 57. Since the conformational change is also observed in pEtxB, which contains an arginine residue at position 13, and hence has only a single histidine residue, protonation of the conserved residue, His 57, may therefore be responsible for the observed conformational change. In the crystal structure of pEtx (Sixma *et al.*, 1993a), His 57 is in a flexible loop, between β4 and α2, which is spatially poorly defined. Upon binding to G_{M1}, this loop becomes fixed (Sixma *et al.*, 1992). To establish whether protonation of the side chain of this residue is responsible for the conformational change observed, a mutant form of hEtxB was generated with a serine residue replacing His 57.

The H57S mutant was expressed and secreted as a pentamer by *Vibrio* sp. 60. Analysis of the H57S mutant by SDS polyacrylamide gel electrophoresis showed that acid-mediated disassembly occurred below pH 2.0, as with the wild type, but that SDS-mediated disassembly showed a shift in pH-dependence from pH 5.0 and below to pH 4.5 and below (data not shown). The modulation of the pH-dependency of the conformational change by this mutation was examined in more detail by studying the pH-dependence of the intrinsic fluorescence of the protein. At pH 7.0 hEtxB (H57S) showed similar fluorescence properties to native hEtxB, with an emission maximum at 346 ± 1 nm when excited at 280 nm. Over the pH range 7.0−3.5, the fluorescence intensity of the mutant decreased with decreasing pH, with no change in λ_{max} (Figure 4). This decrease fitted a model based on a single pK_a-dependent event, with a pK_a of 4.51 ± 0.01 (R^2 = 0.9990); a better fit, with random residuals, was obtained by fitting to a two-pK_a-dependent event with most of the decrease in fluorescence intensity associated with a pK_a of 4.43 ± 0.01 and 10% with a pK_a of 5.64 ± 0.32 (R^2 = 0.9997).

Hence, both by a measure of global stability (susceptibility to SDS-dependent disassembly) and by a measure of local structure (intrinsic fluorescence), the pH-dependent confor-

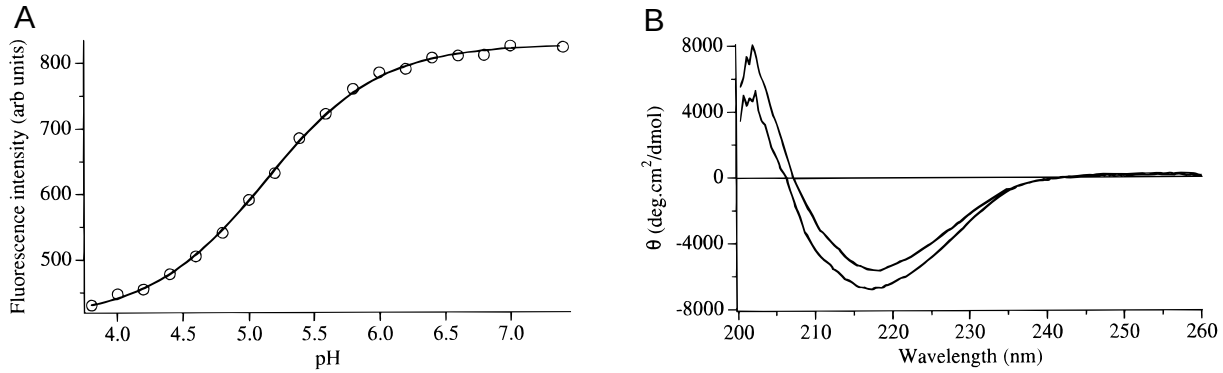

FIGURE 3: pH-dependence of the spectroscopic properties of hEtxB. (A) pH-dependence of the fluorescence intensity of hEtxB (excitation 280 nm, emission 350 nm, slit widths 5 nm). No shift in λ_{max} was observed with pH. Line of best fit to a single pK_a-dependent event. Values shown are the mean ± standard deviation ($n = 3$). (B) pH-dependence of the far-UV circular dichroism spectra of hEtxB. From top to bottom the spectra are in McIlvaine buffer at pH 7.0 and 4.0

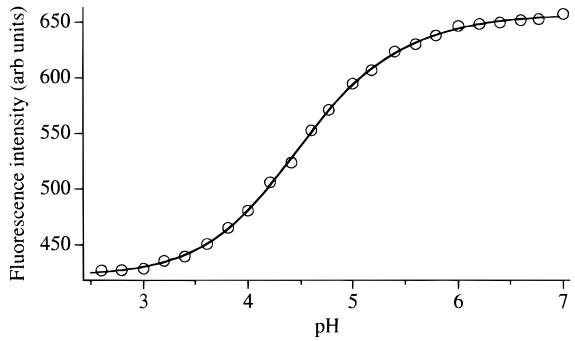

FIGURE 4: pH-dependence of the fluorescence intensity of H57S hEtxB (excitation 280 nm, emission 350 nm, slit widths 5 nm). No shift in λ_{max} was observed with pH. Line of best fit to a single pK_a-dependent event. Values shown are the mean ± standard deviation ($n = 2$).

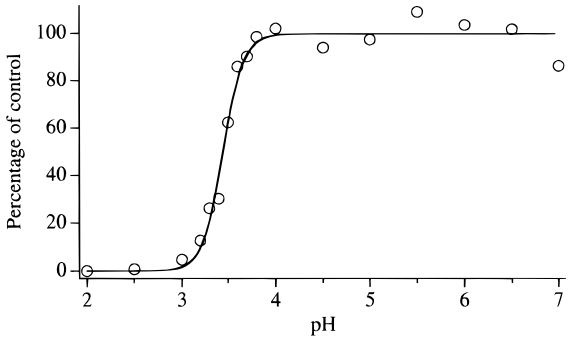

FIGURE 5: pH-dependence of receptor binding determined by G_{M1}-ELISA. hEtxB was incubated at 1 μg/mL in McIlvaine buffer, pH 7.0−2.6, or KCl/HCl, pH 2.5−2.0 with G_{M1} immobilized on an ELISA plate. After receptor binding the amount of pentameric B-subunit bound to the plate was determined by an ELISA using an anti-B specific monoclonal antibody (118-8).

mational change of EtxB is not abolished by the mutation of histidine 57 to serine; however, it is shifted from around pH 5.1 by 0.7 pH unit. This indicates that histidine 57 is not essential for the pH-dependent conformational change, but that it does influence this event.

pH-Dependent Conformational Change Is Not Associated with Changes in Receptor Binding or Dissociation of A−B Interactions

Since a conformational change occurring around pH 5.0 seems to be conserved in the family of AB_5 enterotoxins, it may well have a physiological significance. If the conformational change is linked to toxicity then EtxB must either undergo pH-dependent dissociation from its receptor, G_{M1}, or be able to undergo the conformational change when bound to its receptor, since only this form of the protein is internalized.[3] To determine whether the conformational change is linked to toxicity, the effects on receptor binding were examined. Using a G_{M1}-based ELISA, with the binding step to G_{M1} being performed at varying pH, no pH-dependence of receptor binding was observed over the range pH 7.0−4.0. Lowering the pH further resulted in the abolition of receptor binding in a pH-dependent manner with a midpoint around pH 3.5 (Figure 5). This decrease in binding did not fit to a single pK_a-dependent event, instead it fitted to a co-operative model with the best fit $n = 4$ and pK_a 3.45 ± 0.02 ($R^2 = 0.982$). Receptor binding of hEtxB subjected to buffers over the pH range 7.0−2.0 and subsequently neutralized before the binding step to G_{M1} showed that such prior treatment with acid had no effect on the pH-dependence of receptor binding (data not shown). This indicates that, as observed previously, hEtxB remains pentameric over this pH range (Ruddock *et al.*, 1995) and that abolition of receptor binding around pH 3.5 is fully reversible.

The fact that no decrease in receptor binding was observed around pH 5.0 leads to two possible suppositions; (i) the pH-dependent conformational change is abolished by receptor binding; (ii) the conformational change can still occur, but it has no effect on receptor binding. To investigate this, the pH-dependence of the fluorescence intensity of hEtxB in the presence of G_{M1} was examined.

In the presence of a 40-fold molar excess of G_{M1}, the intrinsic fluorescence of hEtxB at pH 7.0 is quenched, relative to free protein, and there is a blue shift in λ_{max} from 347 to 329 nm. The blue shift seen upon G_{M1} binding, also previously seen for CtxB (De Wolf *et al.*, 1985), arises due to the location of tryptophan in the G_{M1} binding site. Upon binding of G_{M1}, the microenvironment of the tryptophan residue becomes more hydrophobic, resulting in the blue shift in maximal emission wavelength. Over the pH range 7.0−4.0, the fluorescence intensity of hEtxB bound to G_{M1} did not show a pH-dependent change in intensity or λ_{max} (data not shown), consistent with previous work on CtxB (De Wolf

[3] W. I. Lencer, H.M. Webb, and T. R. Hirst, unpublished observations.

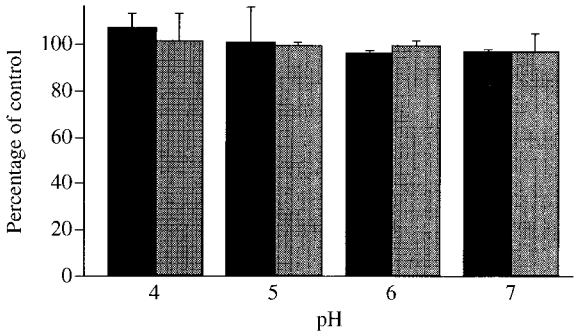

FIGURE 6: pH-dependence of A−B interactions in receptor-bound Etx determined by G_{M1}-ELISA. Etx (1 μg/mL in PBS) was incubated with G_{M1} immobilized on an ELISA plate. After receptor binding samples were incubated in McIlvaine buffer at pH 7.0−4.0 for 15 min and the plates washed. The amount of A- and B-subunit still present on the plate was determined by an ELISA using anti-A (gray bars) and anti-B (black bars) specific antibodies.

et al., 1985). The pH independence of the fluorescence intensity when bound to G_{M1} implies that, when bound to the receptor, the microenvironment of the tryptophan residue does not change in a pH-dependent manner, unlike in non-receptor bound EtxB.

To further examine a putative role for the conformational change in toxicity, the effects of pH on the interaction of the A-subunit with hEtxB were examined by G_{M1}-ELISA. Holotoxin was subjected to buffers of pH 7.0−4.0, either in solution or after binding to G_{M1} immobilized on an ELISA plate. An ELISA was performed on both sets of samples, probing with both anti-A and anti-B antibodies. Since only EtxB binds to G_{M1}, only A-subunit that is still complexed with EtxB will be detected by this method. If there is a pH-dependent disassociation of A from B then there will be a concomitant drop in the amount of A-subunit detected. No pH-dependent decrease in detection of either the B-subunit or the A-subunit was observed for receptor-bound hEtx (Figure 6), implying that A−B interactions are not disrupted by modulation of pH. Similarly, for hEtx in solution, no pH-dependent effects were seen on the proportion of A-subunit associated with pentameric B-subunit (data not shown). We conclude that there is no pH-dependent separation of the A-subunit from B-subunit pentamers over the pH range 4.0−7.0 and that the observed conformational change in the B-subunit at around pH 5.0 is unlikely to be linked to A-subunit release during toxicity.

Molecular Basis of the pH-Dependent Conformational Change

The use of electrophoretic and spectroscopic techniques has demonstrated that a pH-dependent conformational change occurs within the pentameric structure of EtxB. This conformational change results in a small change in CD spectrum, a large quenching of the intrinsic fluorescence and a reduction in the stability of the protein both to elevated temperature and to denaturants. This is consistent with the loss of a significant structure stabilizing element, an alteration of the microenvironment of Trp 88 but with only a minor change in secondary structure. Further analysis of the change in spectral properties and thermal stability indicates that the pH-dependent change fits to a model based on a single pK_a-dependent event, with a pK_a of 5.1.

In order to fully elucidate the physiological role of the pH-dependent conformational change, the molecular basis

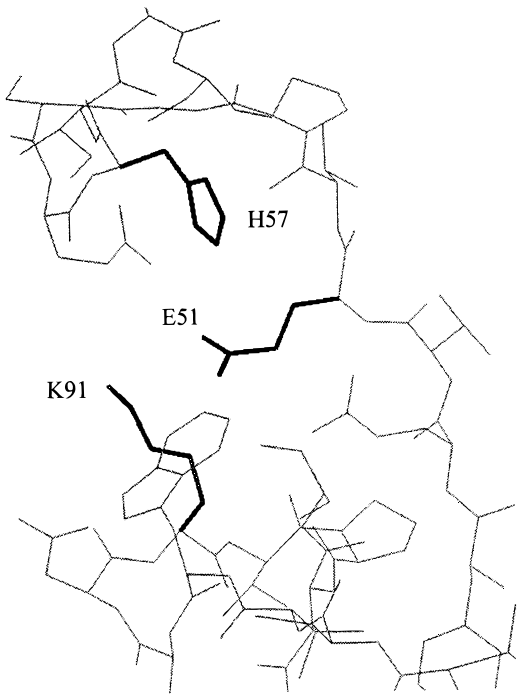

FIGURE 7: Diagrammatic representation of the residues of EtxB thought to be involved in the conformational change around pH 5. The indole side chain of tryptophan 88 (the reporter moiety) can clearly be seen lying above the plane of lysine 91.

of the change must be determined and, if possible, mutants that modulate or abolish the pH-dependence must be generated. pH-dependent conformational changes in proteins arise due to the protonation of side chain groups which may result either in the formation or disruption of salt bridges or in electrostatic repulsion between like-charged species. From the pK_a of the affector and its conservation between hEtxB and pEtxB it was postulated that protonation of the side chain of His 57 was responsible for the conformational change. A mutation of this histidine to serine did not result in the abolition of the conformational change. However, it did result in a shift of the pH-dependence by 0.7 pH units. This indicates that protonation of His 57 influences the conformational change in EtxB, but that it is not essential. The pK_a of the affector group in the H57S mutant is 4.4, indicative of the side chain carboxylate of an aspartic or glutamic acid residue. Hence, in the H57S mutant it is likely that the conformational change results from the protonation of a side chain carboxylate, with the resultant loss of a stabilizing salt bridge. Since the conformational changes in the H57S mutant and wild type EtxB appear to be identical, other than in pK_a, it is likely that protonation of the histidine residue in the wild type protein results in disruption of the same salt bridge. In the crystal structure of pEtx (Sixma *et al.*, 1993a), His 57 is in a flexible loop, between β4 and α2, which is spatially poorly defined. Upon binding to lactose this loop becomes resolved (Sixma *et al.*, 1992) and the imidazole side chain of His 57 is within 4.7 Å of the carboxylate side chain of Glu 51 (Figure 7). In pEtx, Glu 51 forms a salt bridge with Lys 91, linking two three-stranded anti-parallel β-sheets. We postulate that this salt bridge, between Glu 51 and Lys 91, is disrupted by the protonation of His 57 (or directly by protonation of Glu 51 in the H57S mutant) resulting in the observed conformational change. When bound to its receptor, the toxin makes interactions

between the terminal galactose moiety of the receptor and Glu 51, Lys 91, and possibly the residues adjacent to His 57 (Sixma *et al.*, 1992) which could stabilize this region of the protein molecule, thus inhibiting the conformational change, consistent with our observations.

The three residues implicated by this hypothesis in the pH-dependent conformational change, Glu 51, His 57, and Lys 91, as well as the reporter residue Trp 88, are fully conserved within the heat-labile enterotoxin and cholera toxin families. It is therefore likely that the pH-dependent conformational change reported for CtxB occurs via the same mechanism. For verotoxin I it was speculated that the conformational change, which occurs around pH 4.5, resulted from the protonation of either Asp 16 or Asp18 which disrupts a salt bridge formed with Arg 33 (Saleh & Garuépy, 1993). This event was reported by a spatially adjacent tryptophan residue, Trp 34. This situation would be analogous to the H57S mutant reported here. It is also worth noting that just as residues 51, 57, and 91 form part of the receptor binding site of EtxB, it has been reported that Arg 33 and Asp 16 form part of the receptor binding site of verotoxin I (Stein *et al.*, 1992). Hence, it is probable that receptor binding by verotoxin I inhibits the pH-dependent conformational change, just as it is inhibited in EtxB, implying that it is unlikely to have an effect in toxicity.

In an attempt to confirm the proposed molecular basis of the pH-dependent conformational change, we have sought to introduce mutations in Glu 51 and Lys 91. Despite strenuous efforts in a well-established system, no mutations at these positions have been generated. Instead, the mutagenesis generates large numbers of deletion and frame shift mutants, which would tend to suggest that the planned mutants may be toxic to the *E. coli* host in the mutagenesis. In the same system, mutations in 16 other positions have previously been constructed successfully,[4] suggesting that the toxic phenomenon is directly related to mutagenesis of these two sites.

Significance of the pH-Dependent Conformational Change in EtxB

pH-dependent conformational changes, over a similar pH range, have been reported for other members of the AB_5 toxin family (Saleh & Garuépy, 1993; Bhakuni *et al.*, 1991; De Wolf *et al.*, 1985, 1987). Since the pH-dependent conformational change appears to be conserved, it is most likely to be of physiological relevance. It has been speculated for verotoxin I (Saleh & Garuépy, 1993) that the conformational change may be linked to toxicity (via release of the A-subunit) since it occurs at endosomal pH. In this paper we have demonstrated that the interaction of EtxB with its receptor G_{M1} is not affected by the pH-dependent conformational change.[5] Abolition of receptor binding only occurred at lower pH values, with a pK_a of 3.45, which may arise from protonation of either a side chain carboxylate in the protein or the sialic acid of G_{M1} ganglioside. Once bound to its receptor, i.e., in the form in which the toxin is internalized in target cells, the conformational change in EtxB around pH 5.0 is abolished. Further, whether free in solution or bound to G_{M1}, no disruption in A−B interactions occurred over the pH range 7.0−4.0. This evidence suggests that the pH-dependent conformational change is unlikely to have a direct involvement in toxicity. These results complement the recent *in vivo* study on the activity of Ctx in polarized human epithelial T84 cells which showed that neutralization of endosomal pH had no effect on toxin action (Lencer *et al.*, 1996).

There has also been speculation that the conformational change may be linked to secretion of the toxin (Hirst, 1995; Hirst *et al.*, 1995) after the generation of a localised pH gradient at the outer membrane. This speculation is supported by some biophysical evidence since there is a reported pH-dependent increase in hydrophobicity of CtxB (De Wolf *et al.*, 1987) and CtxB has been reported to insert into membranes forming a membrane spanning pore in a pH-dependent manner (Krasilnikov *et al.*, 1991, 1992). It should be noted, however, that the reported change in hydrophobicity, which we have not seen for EtxB, occurs near the pH at which pentameric CtxB dissociates (Ruddock *et al.*, 1995), and the presence of detergent may have resulted in disassembly. In the latter experiments, CtxB was subjected to a pH below that at which it disassociates into its constituent monomers

The molecular basis for the pH-dependent conformational change proposed here has implications for a putative physiological role. The pentameric structure of EtxB is extremely stable (Ruddock *et al.*, 1995). This arises due to the large number of intersubunit interactions, including an extension of the antiparallel β-sheet across the interface and 17 intersubunit salt bridges within the pentamer (Sixma *et al.*, 1993a). Within the monomeric OB fold there is a relatively weak interaction (two hydrogen bonds and one salt bridge) between two parallel β-strands linking the two three-stranded antiparallel β-sheets. The salt bridge is between Glu 51 and Lys 91, and it is the loss of this salt bridge that is proposed to result in the pH-dependent conformational change observed. The loss of this stabilizing salt bridge would allow the potential exposure of the hydrophobic core of the β-barrel. Such exposure would be a prerequisite for the specific secretion from the pathogen by a membrane partitioning mechanism. The fact that verotoxin I, which shares no sequence identity with EtxB or CtxB, appears to be secreted by the same pathway when expressed in *Vibrio* species (Acheson *et al.*, 1993) suggests that these proteins share some common feature which ensures their secretion from members of the *Vibrionaceae*. It is possible that the common property found in these secreted proteins is their ability to undergo a pH-dependent conformational change which increases their hydrophobicity and propensity to partition into a bacterial outer membrane.

[4] H. M. Webb, and T. R. Hirst, unpublished observations.

[5] Though G_{M1} binding is not modulated by pH, over the pH range 7−4, there is evidence that the sugar binding properties of hEtxB are modulated by pH. This arises from pH-dependent binding of hEtxB (but not for a G_{M1} binding mutant G33D hEtxB; Nashar *et al.*, 1996) to a Superdex 75 gel filtration column. (L. W. Ruddock and T. R. Hirst, unpublished results.)

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