Kinetics of Acid-mediated Disassembly of the B Subunit Pentamer of Escherichia coli Heat-labile Enterotoxin

MOLECULAR BASIS OF pH STABILITY*

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The B-subunit pentamer of Escherichia coli heat-labile enterotoxin (EtxB) is highly stable, maintaining its quaternary structure in a range of conditions that would normally be expected to cause protein denaturation. In this paper the structural stability of EtxB has been studied as a function of pH by electrophoretic, immunochemical, and spectroscopic techniques. Disassembly of the cyclic pentameric structure of human EtxB occurs only below pH 2. As determined by changes in intrinsic fluorescence this process follows first-order kinetics, with the rate constant for disassembly being proportional to the square of the H⁺ ion concentration, and with an activation energy of 155 kJ mol⁻¹. A C-terminal deletion mutant, hEtxB214, similarly shows first-order kinetics for disassembly but with a higher pH threshold, resulting in disassembly being seen at pH 3.4 and below. These findings are consistent with the rate-limiting step for disassembly of human EtxB being the simultaneous disruption of two interfaces by protonation of two C-terminal carboxylates. By comparison, disassembly of the B-subunit of cholera toxin (CtxB), a protein which shows 80% sequence identity with EtxB, exhibits a much lower stability to acid conditions; with disassembly of CtxB occurring below pH 3.9, with an activation energy of 81 kJ mol⁻¹. Reasons for the observed differences in acid stability are discussed, and the implications of these findings to the development of oral vaccines using EtxB and CtxB are considered.

Heat-labile enterotoxins from enterotoxigenic Escherichia coli of human (hEtx) or porcine (pEtx) origin are oligomeric proteins comprising one A-subunit, M₁, 27,000, and five B-subunits, M₄, 11,700 (for reviews, see Refs. 1–3). The A-subunits possess ADP-ribosyltransferase activity while the B-subunits act as a carrier system, binding to GM1-ganglioside receptors found ubiquitously on the surfaces of eukaryotic cells (4–6). The B-subunits of hEtx and pEtx show 96% sequence identity to one another and approximately 80% identity to the B-subunit of cholera toxin (CtxB) (7). The crystallographic structure of pEtx revealed that the B-subunits are assembled into a pentameric toroid ring (8). Each B-subunit interacts extensively with its adjacent subunits via multiple hydrophobic, hydrogen bond, and salt bridge interactions. Consequently, the B-pentamers of both cholera toxin and heat-labile enterotoxins are highly stable, maintaining their quaternary structure in a range of conditions that would normally be expected to cause protein denaturation. For example, harsh acidic conditions are required to disassemble hEtxB (9); hEtxB is also stable in the presence of 1% of the ionic detergent sodium dodecyl sulfate (9, 10), in 8 M urea, in 7 M guanidinium chloride and when heated to temperatures ≤80 °C. This stability, as well as their inherent potent immunogenicity and capacity to bind to cell surface receptors, has prompted considerable interest in the potential of CtxB and EtxB as oral delivery vehicles for vaccinating the gut and other mucosa with antigens or epitopes that have been chemically or genetically linked to the B-subunit (for reviews, see Refs. 11 and 12).

The extreme stability of the quaternary structure of hEtxB and the importance of pH stability in withstanding the acidic environment of the stomach (required for effective immunization of the gut mucosal immune system) prompted a systematic study of the disassembly of these proteins at low pH.

Previous studies on pH-dependent stability of oligomeric enterotoxins, in which the change in steady state fluorescence anisotropy of CtxB labeled with dansyl chloride was monitored, have shown that the protein undergoes a progressive dissociation from the pentameric state below pH 4.0 (13, 14). Dissociation was reported to be sensitive to both pH and temperature. This lies within the expected range of stability for oligomeric proteins most of which are fully disassembled at pH 2.6 (15, 16).

This paper shows that nonlabeled, i.e. native, hEtxB pentamers exhibit remarkable acid stability, only undergoing disassembly, to a monomeric state, at pH values below 2.0. Since hEtxB is stable up to pH 11 this represents, to the authors knowledge, the most pH-stable oligomeric complex yet reported. We demonstrate that the stability of hEtxB pentamers in buffers at pH values of 2.0 or above is due to the presence of an intersubunit salt bridge between the C-terminal carboxylate of the B-subunit, Asn-103, and an adjacent subunit in the pentamer. The extreme acid stability of hEtxB pentamers...
trasts with CtxB which is shown to disassemble at pH values below 3.8. The implications of these findings for the use of EtxB or CtxB as potential carriers for oral delivery of antigens are discussed.

**EXPERIMENTAL PROCEDURES**

Purification of hEtxB—A rifampicin-resistant derivative of Vibrio sp. 60 (17) was used as a host for plasmids pMMB68 and pMMB214, which express hEtxB (18) and a single amino acid C-terminal deletion mutant hEtxB214 (19), respectively, under control of an inducible tac promoter. Bacterial strains were grown and EtxB purified by the method described previously (20). Purified hEtxB and hEtxB214 were concentrated by anion exchange chromatography. Protein in 20 mM Tris, pH 7.5 to 2.5 was applied to a 5 ml Resource Q column on an FPLC (Pharmacia). The protein was eluted in 20 mM Tris, pH 7.5, with an increasing linear gradient of 20 mM to 1 M NaCl. The single eluted peak was collected and desalted on a PD10 column (Pharmacia Biotech) equilibrated with phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.4).

When the purified preparations of hEtxB and hEtxB214 were analyzed by SDS-polyacrylamide gel electrophoresis and silver stained, only a single band corresponding to the B-subunits were detected. The yield of purified protein was typically around 10–15 mg/liter of Vibrio sp. 60 culture. Protein concentration was determined using the Bio-Rad protein microassay according to the instructions recommended by the manufacturer. The concentrations of hEtxB and hEtxB214 pentamers were determined using a GM1 ELISA as described previously (20).

Purification of CtxB—0.5-mg aliquots of CtxB (List Biological Laboratories Inc.) were reconstituted in 1.25 ml ELGASTAT UHP purified water. Samples were desalted on a PD10 column (Pharmacia Biotech) equilibrated with 20 mM Tris, 20 mM NaCl, pH 7.5. The desalted protein was applied to a Mono S PC 1.6/5 column on a SMART system (Pharmacia). The protein was eluted in 20 mM Tris, pH 7.5, with an increasing linear gradient of 20 mM to 1 M NaCl. The single eluted peak was collected. When the purified preparation of CtxB was analyzed by SDS-polyacrylamide gel electrophoresis and silver stained, only one band corresponding to the B-subunit was detected. Protein concentration was determined by absorbance at 280 nm, using an extinction coefficient of 9,800 M⁻¹ cm⁻¹, based on the Trp and Tyr content.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed using 14% T and 3% C lanes, or 12% T and 4% C lanes as appropriate. Molecular markers supplied by Bio-Rad were loaded in the outside lanes of each gel. Gels were either stained with 0.05% (w/v) Coomassie Blue R-250 in 40% (v/v) methanol and 10% (v/v) glacial acetic acid or silver stained using the Bio-Rad Silver Stain Kit.

Buffers and Solutions—The buffers used were Mcllvaine buffer (0.2 M disodium hydrogen phosphate, 0.1 M citric acid, pH 7.5 to 2.5) or 0.1 M KC1/HCl (pH 2.5 to 1.0). All buffers were prepared fresh daily using appropriate time drive parameters, duration 3–30 min, sampling intervals 0.5–5 s. Excitation and emission slits were set at 5 nm. The typical lag phase due to manual mixing was 15 s. pH values for 0.1 M KC1/HCl buffers were corrected for the theoretical change due to the addition of small amounts of phosphate-buffered saline and the effects of dilution.

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

**RESULTS**

pH Dependence of Disassembly of hEtxB—Disassembly of the hEtxB pentamer was monitored directly by SDS-gel electrophoresis. This showed a sharp pH threshold for disassembly below pH 2.0, as seen by a transition from the protein running in its pentameric form (around 38 kDa) to its monomeric form (around 12 kDa, Fig. 1A). This was supported by ELISA data based on the pentamer specific property of binding to GM1. At 20°C and pH values of 2.00–7.50, no time dependent changes were seen in the concentration of pentameric hEtxB, as determined by GM1 ELISA. At pH values below 2.00, a time dependent decrease in the concentration of pentameric hEtxB was observed. This decrease followed a first-order process (r² > 0.940) with the rate constant for the observed process increasing with decreasing pH (data not shown).

Spectroscopic parameters were observed to change in parallel with the disassembly process. Circular dichroism spectroscopy showed a large concomitant change in the secondary structure of the protein, consistent with a significant reduction in b sheet (Fig. 1B). At 20°C and pH values of 2.00–7.50 no time dependent changes were seen in the intensity of the fluorescence maxima (excitation 280 nm, emission 347 ± 1 nm) of hEtxB over 1 h (excluding photobleaching). However, at 20°C and pH values of 1.90 or lower, a time dependent decrease in the intensity of the fluorescence maxima was observed. This decrease followed a first-order process (r² > 0.993) with the rate constant for the observed process increasing with decreasing pH (Fig. 2). Over the pH range 1.90 to 1.30 the initial fluorescence intensity (t = 0) was equal to 100 ± 5% of the fluorescence intensity of hEtxB at pH 2.00 and the ratio of the final fluorescence intensity (t = ∞) to the initial fluorescence intensity was 54 ± 2%.

At 20°C, the first-order rate constants for disassembly, as determined by fluorescence and GM1 ELISA, showed a linear dependence on the square of the H⁺ ion concentration, with an apparent first-order rate constant for disassembly of zero at pH = 1.90 ± 0.03 (r² = 0.997, Fig. 3). The identity of the rate constants determined by ELISA and fluorescence confirms that the time dependent fluorescence intensity change is monitoring the disassembly process. At 27°C the first-order rate constants for disassembly, as determined by fluorescence, showed a linear dependence on the square of the H⁺ ion concentration (Fig. 3).

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3) with an apparent first-order rate constant for disassembly of zero at pH 5.192 ± 0.06 ($r^2 = 0.992$). An Arrhenius plot for pH-dependent hEtxB disassembly, at a pH of 1.68, gave a value for the activation energy for disassembly of 155.2 ± 4.1 kJ mol$^{-1}$ ($r^2 = 0.998$, Fig. 4).

Thus both direct and indirect methods for determining the quaternary structure of hEtxB have shown that disassembly from the pentameric state occurs at pH values below 2.0. Measurements of the changes in two independent parameters showed that there was a linear dependence of the first-order rate constant for disassembly on the square of the H$^+$ ion concentration below pH 1.9, with an activation energy of 155 kJ mol$^{-1}$.

A decrease in protein stability at acidic pH values arises from protonation of amino acids in the protein either giving rise to a structure destabilizing interaction, e.g. protonation of a buried side chain, or by the removal of a stabilizing salt bridge. The only groups typically found in proteins with $pK_a$ values around the range at which hEtxB disassembles are C-terminal carboxylates (21). The C-terminal carboxylate has been implicated in an inter-subunit salt bridge with Lys-23 of an adjacent subunit (6). We therefore postulated that the pH-dependent disassembly of hEtxB was directly related to protonation of the C-terminal carboxylate with the resultant loss of the the inter-subunit salt bridge. To test this hypothesis, the pH dependence of disassembly was examined in hEtxB214, a mutant protein in which the C-terminal amino acid Asn-103 had been deleted.

SDS-gel electrophoresis showed a sharp pH dependence for disassembly below pH 3.5, as seen by a transition from the protein running in its pentameric form to its monomeric form (data not shown).
At 25°C and pH values of 3.50–7.50 no time dependent changes were seen in the intensity of the fluorescence maxima (excitation 280 nm, emission 347 nm) of hEtxB214 over 1 h. However, at 25°C a time dependent decrease in the fluorescence intensity maxima was observed at pH values of 3.40 or lower. This decrease followed a first-order process with the rate of the process increasing with decreasing pH. Between pH 3.40 and 2.60 the initial fluorescence intensity (t = 0) was equal to 100 ± 3% of the fluorescence intensity of native hEtxB214 at pH 3.50; but the ratio of the final fluorescence intensity (t = ∞) to the initial fluorescence intensity varied from 68% at pH values of 2.60 to 2.90 to 32% at pH 3.30.

At 25°C the first-order rate constants for disassembly of hEtxB214 showed a linear dependence on the square of the H+ ion concentration, with an apparent first-order rate constant for disassembly of zero at pH = 3.29 ± 0.05 (r² = 0.998, Fig. 5).

An Arrhenius plot for the pH-dependent disassembly of hEtxB214 at a pH of 2.60, chosen to yield similar rate constants for disassembly over the same temperature range as hEtxB, gave a value for the activation energy for disassembly of 127.9 ± 2.2 kJ mol⁻¹ (r² = 0.998, Fig. 4).

Thus, as for wild-type hEtxB, the mutant hEtxB214 pentamer disassembles at acidic pH, with the rate constant for disassembly showing a linear dependence on the square of the H+ ion concentration below a threshold pH. However, the mutant pentamer is clearly destabilized relative to the wild-type. The pH threshold for disassembly was at pH 3.3, some 1.4 pH units higher than that for wild-type, while the activation energy for disassembly was 27 kJ mol⁻¹ less than for the wild-type. We conclude that the presence of terminal residue of wild-type hEtxB is necessary for enhanced acid stability.

pH Dependence of Disassembly of CtxB—It has been reported that dansyl chloride-labeled CtxB undergoes progressive acid-mediated disassembly at pH values below 4.0 (13, 14). To reassess this, time dependent changes in fluorescence intensity of wild-type CtxB was monitored, over a range of pH values. At 25°C and pH values of 3.90–7.50 no time dependent changes were seen in the intensity of the fluorescence maxima (excitation 280 nm, emission 347 nm) of CtxB over 1 h. However, at 25°C and pH values of 3.80 or lower, a time dependent decrease in the fluorescence intensity maxima was observed. Over the pH range 3.70 to 2.80 the initial fluorescence intensity (t = 0) decreased with decreasing pH from 99 to 85% of the fluorescence intensity of native CtxB at pH 3.90 and the ratio of the final fluorescence intensity (t = ∞) to the initial fluorescence intensity was 65.4 ± 0.8%.

The decrease in fluorescence intensity maxima followed a first-order process with the rate constant increasing with decreasing pH. At 25°C the first-order rate constants for disassembly of CtxB showed a linear dependence on the square of the H+ ion concentration, with an apparent first-order rate constant for disassembly of zero at pH = 3.70 ± 0.09 (r² = 0.999, data not shown).

An Arrhenius plot for the pH-dependent disassembly, at a pH of 3.00, gave a value for the activation energy for disassembly of 81.3 ± 1.0 kJ mol⁻¹ (r² = 0.999, data not shown).

Time dependent changes in intrinsic fluorescence showed that disassembly of CtxB pentamers occurs at acidic pH. As for hEtxB and the C terminally truncated hEtxB, disassembly occurred below a pH threshold value, with the rate constant for disassembly being linearly dependent on the square of the H+ ion concentration. However, the CtxB pentamer was significantly less stable than the hEtxB pentamer, since the threshold for disassembly was at pH 3.8, 1.9 pH units higher than that for hEtxB. The activation energy for acid-mediated disassembly of CtxB was 81 kJ mol⁻¹, approximately half the value found for hEtxB disassembly. We conclude that the B-subunit pentamer of E. coli heat-labile enterotoxin exhibits a much higher pH stability than the closely related CtxB pentamer.

**DISCUSSION**

The measurement of time dependent changes in intrinsic fluorescence provides a convenient means of monitoring the kinetics of changes in protein conformation. This method relies upon the occurrence of significant changes in the environment of at least one of the proteins aromatic amino acids. Over the pH ranges that hEtxB, hEtxB214, and CtxB remain stable, their emission maxima were around 347 nm when excited at 280 nm. At these wavelengths the intrinsic fluorescence of these proteins is predominantly due to the single conserved tryptophan at position 88. The crystal structure of pEtxB revealed that this residue is located at the basal plane of the pentamer, forming part of the Gm₁ binding site (6, 8). Disassembly of the pentameric state was found to result in a time, and pH, dependent loss of both Gm₁ binding and a decrease in the intensity of the intrinsic fluorescence of the protein.

The crystal structure of pEtx revealed that the C-terminal carboxylate forms an inter-subunit salt bridge with Lys-23 of the adjacent subunit (6). From the pH dependence for disassembly we postulated that acid-mediated disassembly of hEtxB below pH 2.0 was a direct result of protonation of the C-terminal carboxylate with the resultant loss of the inter-subunit salt bridge. At such low pH values it is probable that the side chain carboxylates of the aspartate and glutamate residues would be protonated leaving the salt bridge involving the C-terminal carboxylate as the only inter-subunit salt bridge. This would be protonated leaving the salt bridge involving the C-terminal carboxylate as the only inter-subunit salt bridge.
Hence acid-mediated disassembly of hEtxB is proposed to occur with the loss of the last, structure stabilizing, inter-subunit salt bridge between the C-terminal carboxylate and Lys-23.

Consistent with this hypothesis was the finding that the C-terminal deletion mutant hEtxB214 disassembles at much higher pH values. Previous work (18) has shown that there were no apparent differences between hEtxB and hEtxB214 with regard to SDS susceptibility, in vivo assembly, or G\textsubscript{M1} binding, hence it is likely that both proteins have nearly identical quaternary structures. From the crystal structure of pEtx it is unlikely that a salt bridge could be formed between the C-terminal carboxylate and Lys-23. Hence acid-mediated disassembly of hEtxB is proposed to occur with the loss of the last, structure stabilizing, inter-subunit salt bridge.

CtbX was found to disassemble at higher pH values than hEtxB and to have a significantly lower activation energy for disassembly (81 kJ mol\textsuperscript{-1}, c.f. 151 kJ mol\textsuperscript{-1}). Since CtbX and hEtxB share 81% sequence identity (over 90% similarity) and x-ray crystallographic analyses of pEtx and CtbX have revealed that the B-subunits possess the same structural fold, it is reasonable to presume that the B-pentamers of the enterotoxin family have near identical tertiary and quaternary structures (3). Indeed, all of the amino acids implicated in side chain inter-or intra-subunit salt bridges and those implicated in side chain inter-subunit hydrogen bonding are fully conserved. Thus, the differences in pH-dependent stability of CtbX and hEtxB are likely to arise from differences in residues that are protonatable. The two changes in such residues are Tyr-18 and Asn-94 in hEtxB, which are both histidines in CtbX. Neither of these residues are particularly deeply buried in the crystal structure of pEtx nor are they in regions of high charge density. However, the side chains of the corresponding residues in pEtx are within 3 Å of each other. Hence in CtbX, at pH values below pH 4, where histidines are likely to be protonated, there will be electrostatic repulsion between these two residues. This repulsion may be the cause of the much lower stability of CtbX compared with hEtxB.

hEtxB, hEtxB214, and CtbX all show a linear dependence of the first-order rate constant for disassembly on the square of the H\textsuperscript{+} ion concentration. This dependence implies a requirement for two protonation events in the rate-limiting step. This is consistent with protonation of the C-terminal carboxylate being the only requirement for acid-mediated disassembly, if two subunits require this group to be protonated simultaneously for disassembly to occur. This requirement for two groups to be protonated, i.e. for two inter subunit interfaces to be disrupted, can be rationalized due to the cyclic nature of the quaternary structure of hEtxB (see Fig. 7). If a single interface is broken in a cyclic pentamer the structure is still pentameric whereas if two interfaces are broken simultaneously smaller structures, e.g. a trimer and a dimer, are formed. These smaller oligomeric structures are probably very unstable in the conditions in which they are formed, since only a single interface need be disrupted for their further disassembly, and are likely to be rapidly broken down into monomeric units.

The demonstration that hEtxB is more acid-stable than CtbX has important implications for the use and development of these proteins as oral vaccine components and vaccine delivery vehicles. Disassembly of B-subunit pentamers significantly reduces their capacity to induce an anti-toxin immune response because of the loss of their G\textsubscript{M1}-binding property, which normally aids uptake and presentation to the immune system.\textsuperscript{5} The effectiveness of regimes to neutralize stomach acid when CtbX or EtxB are given perorally as components of oral vaccines (22, 23) is not known and may vary from individual to individual. The findings reported here suggest that the EtxB pentamer should be better at withstanding the acidic environment of the stomach and thus be a more effective oral immunogen.

CtbX and EtxB are also being evaluated as oral delivery vehicles for vaccinating the gut with antigens or epitopes that have been genetically or chemically fused to the B-subunit (12, 24–29). Fusions to EtxB have been exclusively designed such that epitope or antigen extensions are present at the C terminus of the B-subunit (12). This reflects the availability of conventional restriction sites, the initial demonstration that the C terminus of EtxB may be extended without interfering with B-subunit assembly or G\textsubscript{M1} binding, and the choice of whether to target fusion proteins to the cytoplasm or periplasm. The finding reported here that the acid stability of EtxB is dependent on an inter-subunit salt bridge involving the C-terminal carboxylate would suggest that the recombinant EtxB-fusion proteins (which have C-terminal extensions) should disassemble at higher pH values than the wild-type EtxB pentamer. Indeed, we have found that an (Asn-Ala-Asn-Pro)\textsubscript{3} extension at the C terminus of hEtxB (30) results in a pentameric protein which disassembles below pH 3.52, with an activation energy

\textsuperscript{5} R. F. L. James, T. O. Nashar, T. Amin, and T. R. Hirst, unpublished observations.
for disassembly of 140 kJ mol$^{-1}$. Thus, our findings on the molecular basis of the acid stability of EtxB should guide the design of new conjugates which ensure retention of the native C-terminal carboxylate.

The work detailed here on acid-mediated disassembly not only allows insight into the molecular basis of the extreme stability of the quaternary structure of hEtxB and the rationalization of chimeric vaccine design, but also forms the basis for further work on the reassembly pathway of this remarkable protein.

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