Orientation within the Exosporium and Structural Stability of the Collagen-Like Glycoprotein BclA of *Bacillus anthracis*

Jeremy A. Boydston, Ping Chen, Christopher T. Steichen and Charles L. Turnbough Jr.


Updated information and services can be found at:
http://jb.asm.org/content/187/15/5310

**REFERENCES**

This article cites 31 articles, 17 of which can be accessed free at:  http://jb.asm.org/content/187/15/5310#ref-list-1

**CONTENT ALERTS**

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article),  more»
Orientation within the Exosporium and Structural Stability of the Collagen-Like Glycoprotein BclA of \textit{Bacillus anthracis}

Jeremy A. Boydston, Ping Chen, Christopher T. Steichen, and Charles L. Turnbough, Jr.*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Received 16 March 2005/Accepted 22 April 2005

\textit{Bacillus anthracis} spores, which cause anthrax, are enclosed by an exosporium consisting of a basal layer and an external hair-like nap. The filaments of the nap are composed of BclA, a glycoprotein containing distinct N-terminal (NTD) and C-terminal (CTD) domains separated by an extended collagen-like central region. In this study, we used immunogold electron microscopy to show that the CTD of BclA forms the distal end of each filament of the hair-like nap, indicating that the NTD is attached to the basal layer. Ten randomly chosen anti-BclA monoclonal antibodies, raised against spores or exosporium, reacted with the CTD, consistent with its exterior location. We showed that recombinant BclA (rBclA), encoded by the \textit{B. anthracis} Sterne strain and synthesized in \textit{Escherichia coli}, forms a collagen-like triple helix as judged by collagenase sensitivity and circular dichroism spectroscopy. In contrast, native BclA in spores was resistant to collagenase digestion. Thermal denaturation studies showed that the collagen-like region of rBclA exhibited a melting temperature ($T_m$) of 37°C, like mammalian collagen. However, rBclA trimers exhibited $T_m$ values of 84°C and 95°C in buffer with and without sodium dodecyl sulfate, respectively. CTD trimers exhibited the same $T_m$ values, indicating that the high temperature and detergent resistances of rBclA were due to strong CTD interactions. We observed that CTD trimers are resistant to many proteases and readily form large crystalline sheets. Structural data indicate that the CTD is composed of multiple beta strands. Taken together, our results suggest that BclA and particularly its CTD form a rugged shield around the spore.

\textit{Bacillus anthracis}, the causative agent of anthrax, is a gram-positive, rod-shaped, aerobic bacterium that forms endospores (or spores) when deprived of certain essential nutrients (6, 22). Spores are dormant and are capable of surviving in the soil and other adverse environments for many years (20). When spores encounter an aqueous environment containing appropriate nutrients, they can germinate and grow as vegetative cells (14). The mature \textit{B. anthracis} spore contains a central, genome-containing compartment called the core and three prominent protective layers called the cortex, coat, and exosporium (9). These layers are synthesized in the later stages of spore development (9, 13). The cortex, which is adjacent to the core, is composed of a thick layer of peptidoglycan (7). The exosporium is a loose-fitting, balloon-like layer that encloses the spore and is composed of perhaps as many as 20 different proteins in tight and loose associations (24, 28, 33).

As the outermost layer, the exosporium serves as the primary permeability barrier of the spore and as the source of spore surface antigens (9, 28). In addition, the exosporium interacts with the soil environment, with spore-binding cells in the mammalian host, and with host defenses. The exosporium is divided into two regions, a paracrystalline basal layer and an external hair-like nap (9). Although the components of the basal layer are not well characterized, a single glycoprotein was identified as the structural component of the filaments of the hair-like nap (31, 32). This protein contains three domains: a 38-amino-acid amino-terminal domain (N-terminal domain [NTD]), apparently processed to 19 amino acids; a central, collagen-like region containing Xaa-Yaa-Gly (XXG) repeats; and a 134-amino-acid carboxy-terminal domain (CTD) (28, 31). Accordingly, this protein was called BclA for \textit{Bacillus} collagen-like protein of \textit{anthracis} (31). The number of XXG (mostly PTG) repeats found in different naturally occurring \textit{B. anthracis} strains varies from 17 to 91 (28, 31, 32). The length of this region is proportional to the length of the hair-like nap, which varies from 140 to 608 Å (32). The collagen-like region of BclA appears to be nearly fully extended (15) and extensively glycosylated (4). Finally, it was shown that BclA is the immunodominant protein on the spore surface, suggesting a role for this protein in spore-host interactions (28).

In the present study, we examine the orientation of BclA in the exosporium. We use a CTD-specific monoclonal antibody (MAb) and immunogold electron microscopy to demonstrate that the CTD of BclA forms the distal end of each exosporium filament. We also demonstrate that BclA forms a trimeric, collagen-like structure by using gel electrophoresis, collagenase digestion, and circular dichroism spectroscopy. Finally, we provide evidence that trimerization of BclA is facilitated by highly stable CTD interactions.

MATERIALS AND METHODS

\textit{B. anthracis} strains. The Sterne 34F2 veterinary vaccine strain of \textit{B. anthracis}, which was used as the wild-type strain in this study, was obtained from the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD. The Sterne strain is not a human pathogen because it lacks a plasmid (i.e., pXO2) necessary to produce the capsule of the vegetative cell (10). Three variants of the Sterne strain were used in this study. Two variant strains containing different bclA deletions, designated $\Delta bclA$ and 1/2 CLR, were constructed by allelic replacement as previously described (4). The $\Delta bclA$ mutation deletes most of the \textit{bclA} gene (i.e., codons 27 to 382 of 400), and the 1/2 CLR mutation reduces the

* Corresponding author. Mailing address: UAB Department of Microbiology, BBRB 409, 1530 3rd Ave. S, Birmingham, AL 35294-2170. Phone: (205) 934-0289. Fax: (205) 975-5479. E-mail: ChuckT@uab.edu.
number of XXG repeats in the collagen-like region from the normal 76 to 37.

The concentration of highly purified rBclA was determined by acid hydrolysis and quantitative amino acid analysis, which was performed by the Molecular Structure Facility of the University of California, Davis. The concentration of rBclA in cell extracts was estimated from Coomassie brilliant blue staining of proteins separated by SDS-PAGE, with purified rBclA used as a standard. The concentrations of purified CTD of BclA and GST-CTD were also estimated from Coomassie brilliant blue staining as described above, except that Bio-Rad broad-range molecular weight markers were used as standards.

RESULTS

Identification of the BclA domain recognized by anti-BclA MAb. Our first goal in this study was to determine the orientation of BclA within the hair-like nap of the exosporium. Our plan was to identify the distal end of the hair-like filaments on intact spores by immunogold electron microscopy using a primary mouse MAb capable of binding the distal domain of BclA and a secondary anti-mouse IgG linked to a gold bead. To find a primary MAb with the appropriate reactivity, we examined 10 previously described unique mouse anti-BclA MABs for

Electron microscopy. All steps in sample preparation were performed at room temperature unless indicated otherwise. For transmission electron microscopy, spores (10^9) were fixed for 24 h at 4°C in a solution of 1.25% formaldehyde, 4% paraformaldehyde, and 2% (vol/vol) dimethyl sulfoxide in phosphate-buffered saline (PBS) (27). The spores were thoroughly rinsed in PBS and stained with 1% osmium tetroxide for 3 h at 4°C. After rinsing, the spores were further stained with 1% tannic acid for 20 min and rinsed twice in PBS and then in distilled water. The spores were dehydrated in a graded ethanol series including 50% (vol/vol) ethanol, 75% ethanol containing 1% uranyl acetate (for 1 h), 70% ethanol (twice), 95% ethanol, and 100% ethanol (four times), which was followed by two 30-min rinses in propylene oxide. The spores were embedded in Spurr's low-viscosity resin (Electron Microscopy Sciences). Thin (100-nm) sections of polymerized resin were placed on copper grids and stained with 1% alcoholic uranyl acetate and Reynolds' lead citrate (25) for 9 and 5 min, respectively. Sections were examined under a Hitachi 7000 electron microscope operated at 57 kV.

For immunogold electron microscopy, spores (10^9) were fixed for 30 min on ice in a solution of 4% paraformaldehyde and 0.1% (vol/vol) glutaraldehyde in PBS. After two 5-min washes in PBS, the spores were dehydrated in a series of 15-min incubations in 50%, 75%, 95%, and (twice in) 100% ethanol. The spores were infiltrated for 1 h in a 1:1 mixture of LR white resin (London Resin Company) and 100% ethanol and then infiltrated with LR white resin (London Resin Company). The spores were infiltrated for 2 h in fresh LR white resin and resuspended again in fresh resin. This suspension was incubated at 42°C for 48 h to embed the spores in polymerized resin. Thin (100-nm) sections of this sample were placed on nickel, carbon-coated formvar grids. Nonspecific binding sites on the grids were blocked by a 30-min incubation in TBS buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 20 mM NaN_3) containing 1% bovine serum albumin (BSA). The grids were then incubated in 1:200 dilution of goat anti-mouse IgG plus IgM (heavy plus light chains) labeled with 10-nm gold beads (Amersham Biosciences). The sections were washed in TBS buffer and then washed in PBS, fixed for 2 min with 0.1% glutaraldehyde in PBS, washed in water, lightly stained with 2% uranyl acetate and Reynolds' lead citrate, and analyzed as described above.

Collagenase digestion. Protein and spore samples were digested with type III fraction A collagenase from Clostridium histolyticum (Sigma). This collagenase preparation is highly purified and substantially free of nonspecific proteases. Reaction mixtures (50 μl) contained 50 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.4), 0.36 mM CaCl_2, and collagenase and substrate as indicated. Reaction mixtures were incubated for 90 min at 37°C, and digestion was stopped by the addition of a 1/4 volume of 5× sample buffer and boiling for 8 min.

CD spectroscopy. Circular dichroism (CD) analysis was performed with an AVIV model 62DS spectropolarimeter (Lakewood, NJ) equipped with a single cell thermostatic device. Protein samples were dissolved in PBS, pH 7.4. Wave-length scans were performed in triplicate using a 1.0-mm-path-length cuvette, and data were collected every 0.5 nm with a 1-nm bandwidth and a 16-s averaging time. Protein-unfolding curves were performed in a 1.0-cm-path-length cuvette at 220 nm. Data were collected stepwise every 1 nm using a 3-nm bandwidth, a 60-s averaging time, and a 60-s equilibration time. All scans were baseline corrected using a PBS blank.
MAbs raised against B. anthracis binding to a particular domain of BclA. We examined five polypeptide chain migrated with an apparent mass of 70 kDa as previously described (28). This aberrantly slow mobility is typical of collagen-like proteins (18). (B) Purified rBclA, GST-CTD fusion protein, and GST were separated by SDS-PAGE as described above (A), the proteins were electrophoretically transferred to a nitrocellulose membrane, and the membrane was immunoblotted using EF12 as the primary antibody.

binding to a particular domain of BclA. We examined five MAbs raised against B. anthracis spores and five raised against purified exosporium (28). We began our search by screening the MAbs for binding to the CTD of BclA. As a source of CTD, we used purified GST-CTD fusion protein. (Purified CTD was not used in this experiment to avoid problems associated with its low solubility.) The results showed that every MAb reacted with rBclA, as previously shown, and with the GST-CTD fusion protein. An immunoblot with MAB EF12, which is typical, is shown in Fig. 1. To verify that the MAbs were binding to the CTD portion of the fusion protein, we demonstrated that EF12 and all of the other MAbs failed to react with purified GST (Fig. 1 and data not shown). Thus, we were limited in our attempt to attach gold beads to the distal end of exosporium filaments to a MAb that binds the CTD of BclA, which proved to be sufficient.

Localization of the CTD of BclA to the distal end of the filaments in the exosporium hair-like nap. To aid in locating the CTD of BclA within the filaments of the hair-like nap, we examined spores of the wild-type Sterne strain and also mutant spores displaying a short or long version of BclA. The 400-amino-acid wild-type BclA protein contains a collagen-like region with 76 XXG repeats (Fig. 2). In comparison, the short and long mutant BclA proteins contain 37 and 107 XXG repeats longer than the wild-type protein, respectively (Fig. 2). Transmission electron microscopy revealed that the exosporium filaments on spores were not found further from the basal layer than the length of the BclA protein, which presumably reflects filament slanting. Beads that appeared to be associated with spores were not found further from the basal layer than the length of the BclA protein. These data clearly place the CTD epitopes for MAb binding (Fig. 4).

Confirmation of the collagen-like triple-helical structure of rBclA by collagenase digestion. All members of the collagen family of proteins contain a domain with multiple adjacent repeats of a proline-rich tripeptide with glycine in every third position. Typically, the triplet repeat is PXG. The repeat region forms the characteristic collagen triple helix (8). The central region of BclA is composed of a long run of XXG (mostly PTG) repeats (Fig. 2), and it has been assumed that this region forms a collagen-like triple helix (31). The native collagen triple helix is resistant to most proteases; however, the triple helix is degraded with high specificity by collagenases (8). Recent studies indicate that collagenases bind the collagen triple helix, locally unwind this structure, and then hydrolyze specific peptide bonds (3). The type III, fraction A collagenase from C. histolyticum degrades the helical region in native collagen by preferentially cleaving the X-G bond of the sequence PXGP (11). Target sequences for this bacterial collagenase are

FIG. 1. Binding of MAb EF12 to rBclA and to the CTD of BclA. (A) Similar amounts of purified rBclA (39 kDa), GST-CTD fusion protein (40 kDa), and GST (27 kDa) were separated by SDS-PAGE and stained with Coomassie brilliant blue. Note that the rBclA polypeptide chain migrated with an apparent mass of ~70 kDa as previously described (28). This aberrantly slow mobility is typical of collagen-like proteins (18). (B) Purified rBclA, GST-CTD fusion protein, and GST were separated by SDS-PAGE as described above (A), the proteins were electrophoretically transferred to a nitrocellulose membrane, and the membrane was immunoblotted using EF12 as the primary antibody.

FIG. 2. BclA collagen-like regions of the wild-type Sterne strain and of bclA mutant strains that produce the short and long versions of BclA. The short and long versions of BclA are the consequence of an internal deletion and an insertion in the bclA gene, respectively. Wild-type BclA contains 400 amino acids, with residues 39 to 266 included in the collagen-like region. The deletion and insertion mutations result in BclA proteins with collagen-like regions that are 39 XXG repeats shorter and 31 XXG repeats longer than the wild-type protein, respectively.
located throughout the central region of BclA. Thus, this enzyme could be used as a probe for collagen-like structure in BclA.

Accordingly, we treated a partially purified sample of rBclA with increasing concentrations of collagenase from *C. histolyticum*. The substrate sample contained approximately 20 μg of rBclA and low levels of many *E. coli* proteins, which served as controls for the specificity of the digestion. The collagenase concentration in six reaction mixtures was serially increased from $4 \times 10^{-4}$ to 40 U/ml (i.e., 10-fold increases). All reactions

---

**FIG. 3.** Electron micrographs of thin sections of wild-type and mutant *B. anthracis* (Sterne) spores focusing on the exosporium basal layer and attached hair-like nap. The mutant spores were produced by two strains with different internal *bclA* deletions and another strain containing an insertion in the *bclA* gene. One deletion (Δ*bclA*) removes essentially the entire *bclA* gene, whereas the other deletion removes about one-half of the normal 76 XXG codons and encodes the short BclA protein. The insertion extends the triplet repeat region to 107 XXG codons, and this mutant *bclA* gene encodes the long BclA protein. The filament lengths of the naps on the spores are indicated in parentheses. In each panel, an arrowhead points to the exosporium basal layer. Bars, 1,000 Å.

**FIG. 4.** Localization of the CTD of BclA to the distal end of the filaments in the exosporium hair-like nap. Immunogold electron microscopy was used to locate the CTD of BclA on wild-type spores and on mutant spores displaying the short or long version of BclA. The filaments of the exosporium hair-like naps are not visible in these electron micrographs due to light staining required for immunogold tagging. Electron-dense gold beads (10 nm in diameter) indicate the position of the CTD of BclA and the distance between the CTD and the basal layer of the exosporium. No gold beads were found attached to control Δ*bclA* spores. In each panel, an arrowhead points to the exosporium basal layer. Bars, 1,000 Å.
rBclA produced a doublet of The extent of digestion was proportional to the amount of firming the presence of the collagen-like triple helix (Fig. 5A). showed that rBclA was indeed digested by collagenase, con-

primary antibody. Examination of the Coomassie-stained gel by SDS-PAGE and immunoblotting using MAb EF12 as the volume from each reaction mixture was removed for analysis Materials and Methods. After stopping the reactions, an equal concentrations on BclA contribute to collagenase resistance.

**BclA on spores is resistant to collagenase digestion.** We also examined the ability of the C. histolyticum collagenase to cleave native BclA present on the B. anthracis spore surface. Sterne spores (10^8) were treated with a high level of collagenase (1,000 U/ml or 2 mg/ml) for 1 h under standard reaction conditions. A control reaction lacking collagenase was also performed. After the reactions were complete, the reaction mixtures were boiled for 8 min in sample buffer and centrifuged to remove insoluble material. The sample supernatants were then examined for the presence of the CTD of BclA by immunoblotting as described above. No CTD fragments were detected in either the collagenase-treated or the control sample, while similar levels of full-length BclA were detected in both samples (data not shown). In another experiment, collagenase-digested and control Sterne spores were examined for binding of fluorescantly labeled MAb EF12 by flow cytometry (34). All spores exhibited extensive binding of this CTD-specific MAb, and the number of MAbs bound per spore was not measurably altered by collagenase treatment (data not shown).

The collagen-like domain of BclA on Sterne spores is glycosylated at multiple sites with a pentasaccharide (4), modification that could protect against collagenase cleavage. Spores produced by a ΔrmlD mutant strain, unable to synthesize 1-rhamnose, are glycosylation deficient; i.e., only the reducing-end N-acetylgalactosamine remains of the pentasaccharide (4). To determine if glycosylation-deficient spores were susceptible to collagenase digestion, the experiments performed above with wild-type spores were repeated with ΔrmlD spores. The results were essentially the same as those obtained with wild-type spores: no collagenase digestion of BclA was detected by immunoblotting or by flow cytometry (data not shown). Thus, it appeared that wild-type and glycosylation-deficient BclA proteins were highly resistant to collagenase when normally incorporated into the exosporium. It should be noted that the experiment with ΔrmlD spores did not exclude the possibility that the remaining N-acetylgalactosamine residues on BclA contribute to collagenase resistance.

**Collagen-like circular dichroism spectrum and thermal denaturation of the triple helix of rBclA.** Collagen triple helices have characteristic CD spectra with a positive ellipticity maximum at 220 nm (2). We analyzed a highly purified sample of rBclA by CD spectroscopy at 4°C. The results showed a spectrum that strongly resembled that of the collagen triple helix (36) (Fig. 6A, solid line). When a sample of purified rBclA was heated to 50°C and a CD spectrum was recorded at this temperature, the characteristic features of the collagen triple helix were absent. These features were replaced by a spectrum resembling that of a random coil (Fig. 6A, broken line), indicating that the triple helix had unfolded (36).
To examine the thermal denaturation of the triple helix in detail, we monitored the CD of purified rBclA at 220 nm as a function of increasing temperature from 20 to 50°C (Fig. 6B). The triple helix appeared to unfold within an approximately 10°C range, with a melting temperature ($T_m$) of 37°C. The thermal unfolding of the rBclA triple helix was reversible. When rBclA was unfolded at 50°C and then incubated at 4°C, complete renaturation occurred within 2 h as judged by a CD spectrum that was virtually identical to that of the unheated protein (data not shown).

Trimerization of rBclA through CTD interactions. Collagen forms trimers that are often nucleated by a noncollagenous CTD (1). To examine the ability of rBclA to assemble into trimers, we dissolved 30 g of purified protein in 50 l of sample buffer. This sample was split in half, with one half boiled for 8 min. The two samples were analyzed by SDS-PAGE. Boiled rBclA ran as a single species with an apparent mass of 70 kDa (Fig. 7). This species corresponded to a single polypeptide chain of rBclA. Unboiled rBclA ran as a single species with an apparent mass of 200 kDa (Fig. 7). This result indicated that unboiled rBclA formed a trimer, which was stable in sample buffer containing 2% SDS and during gel migration in the presence of 0.1% SDS.

To assess the contribution of the CTD of BclA to trimer formation, we repeated the experiment described above with either GST-CTD (40 kDa) or CTD (16 kDa with the His6 tag). Approximately 4 g of purified protein was dissolved in 50 l of sample buffer in each case. The results showed that each boiled protein ran in the gel predominantly as one species, with an apparent mass that corresponded closely to that of a single polypeptide chain (Fig. 7). On the other hand, each unboiled protein ran with an apparent mass that was three times that of a single polypeptide chain (Fig. 7). Thus, both GST-CTD and CTD formed SDS-resistant trimers, as described for rBclA. These results indicate that the CTD of BclA contributes to trimer formation.

Stability of rBclA and CTD trimers. To examine the stability of the trimers formed by rBclA and CTD, we measured their dissociation with increasing temperature. Each protein was dissolved in sample buffer and also in PBS. Equal samples of these solutions were incubated at 25, 60, 70, 80, 90, or 100°C. After 8 min of incubation, the samples were quickly cooled to room temperature. The samples in PBS were made 1× in sample buffer, and all samples were loaded (within several minutes after incubation) onto a gel for analysis by SDS-PAGE. Proteins in the gel were stained with Coomassie brilliant blue, which revealed that trimers dissociated to monomers only and that monomers did not appear to reform into trimers during analysis (data not shown). The stained proteins were quantitated by densitometry. The results indicated that in sample buffer, virtually all trimers formed by rBclA and CTD remained intact at up to 70°C (Fig. 8). At higher temperatures, trimers of each protein dissociated with similar kinetics, and dissociation was complete at 90°C. Each trimer in sample buffer exhibited a $T_m$ of ~84°C. The trimers of rBclA and CTD were even more stable in PBS. These trimers remained completely intact at up to 80°C and were slightly dissociated at 90°C but were completely dissociated at 100°C (Fig. 8). Each trimer in PBS exhibited a $T_m$ of 95°C. These results demonstrate that both rBclA and CTD form extremely heat-stable trimers and that the stability of the trimers appears to be due entirely to CTD interactions.

In addition to heat sensitivity, we examined the susceptibility of CTD trimers to proteolytic digestion. Dilute samples of CTD trimers were subjected to prolonged exposure to high
concentrations of a particular protease (i.e., trypsin, chymotrypsin, proteinase K, and elastase) or protease mixture (i.e., pronase E). Reaction conditions were those recommended for optimal digestion by the commercial suppliers of the proteases. Except for their amino-terminal His6 tags, the CTD trimers were completely resistant to all the proteases examined as judged by SDS-PAGE (data not shown).

Formation of crystals by CTD. We observed that purified CTD precipitated from concentrated solutions soon after purification. (Note that the experiments described above were performed with soluble CTD.) Phase-contrast microscopy revealed that the precipitated material was crystalline. The crystals formed regular and irregular hexagons, which were as wide as 20 μm (Fig. 9). However, these crystals were very thin and quite fragile. The crystals bound fluorescently labeled MAb EF12, with binding being much more pronounced on the edges of the crystals (data not shown). No crystal formation was observed with rBclA or GST-CTD, suggesting that the non-CTD regions of these proteins suppressed crystallization in solution.

DISCUSSION

Previous studies indicated that the filaments of the hair-like nap of the B. anthracis exosporium are formed by the elongated collagen-like glycoprotein BclA (4, 28, 31, 32). BclA contains three domains: an NTD; a central, highly extended collagen-like region; and a CTD. A primary goal of this study was to determine the orientation of the tripartite BclA protein within each filament of the hair-like nap. Using immunogold electron microscopy, we showed that the CTD of BclA forms the distal end of each filament. This orientation is consistent with the observation that the vast majority, if not all, of anti-BclA MAbs raised against B. anthracis spores or purified exosporium react with the CTD of BclA. The proposed orientation of BclA also suggests that the NTD of BclA forms the proximal end of each filament and contains the site of attachment to the basal layer of the exosporium. This model is supported by preliminary studies that indicate that deletion of the NTD of BclA prevents attachment of the truncated protein to the basal layer (K. L. Roberts and C. L. Turnbough, Jr., unpublished data).

As observed with other bacterial collagen-like proteins (23, 36), the rBclA encoded by the Sterne strain of B. anthracis and synthesized in E. coli forms trimers. These trimers are sensitive to collagenase and exhibit a circular dichroism spectrum typical of collagen, indicating the formation of a collagen-like triple helix (2, 36). Thermal denaturation studies revealed a $T_m$ of 37°C for the collagen-like region of rBclA, which is typical for mammalian collagen (17). However, rBclA trimers are much more stable than mammalian collagens. These trimers displayed $T_m$ values of 95°C in PBS and 84°C in sample buffer containing 2% SDS (when incubated for 8 min). Essentially the same stability was displayed by trimers formed by just the CTD of BclA, which indicates that the highly stable structure of rBclA is due to strong CTD interactions. These strong CTD interactions probably assist in the spontaneous refolding of the collagen-like region of rBclA after denaturation at 50°C, which was observed in this study. In fact, many types of collagen use noncollagenous CTD interactions to initiate formation of their triple-helical structure (1, 5). Taken together, our observations suggest that each filament of the hair-like nap on spores is a BclA trimer.

The formation of stable trimers of the CTD of BclA is likely to occur through associations between beta strands. According to the structure predicted by the BioMolecular Engineering Research Center Protein Sequence Analysis server, each CTD monomer is formed by a series of approximately 12 beta strands (29, 30, 35). trimers formed by proteins with similar secondary structures are often extremely stable (12, 19). The predicted structure for the CTD of BclA is consistent with a recent report describing the preliminary characterization of
the crystal structure of trimers formed by a recombinant BclA encoded by \textit{B. anthracis} strain CEB 9732 (26). The BclA encoded by this strain contains only 20 XXG repeats in its collagen-like region, compared to 76 XXG repeats in the BclA encoded by our Sterne strain. In the crystal structure of the CEB 9732 rBclA, only the CTD is ordered, and its structure is determined to 2Å resolution. As we observed with the Sterne rBclA, the CEB 9732 rBclA forms trimers in solution that are sensitive to collagenase digestion (26).

We made three other observations in this study that may be related to the function of BclA on the spore surface. We found that native BclA in spores, even when glycosylation deficient, is not susceptible to collagenase digestion. We showed that trimers of the CTD of BclA are extremely resistant to several different proteases. We observed that large crystalline sheets were formed in concentrated solutions of purified CTD. These properties, along with the resistance of CTD trimers to high temperatures and detergent concentrations, suggest that the CTD of BclA forms a rugged permeability barrier or shield around the spore. This shield may explain a previous observation that the presence of BclA on spores severely limits the viability and virulence. Although such roles remain to be demonstrated, it seems likely that the properties of BclA described in this study contribute to the fate of \textit{B. anthracis} spores whether they are lying dormant in the soil or initiating an infection within tissues of a mammalian host.

ACKNOWLEDGMENTS

We thank John Kearney for generously providing MAbs, Leigh Milican of the UAB High Resolution Imaging Facility for valuable assistance with electron microscopy, Mike Jablonsky and Don Muccio for their help with circular dichroics spectroscopy, and John Trombley and James Daubenspeck for performing protease digestions. This work was supported by NIH grant AI50566 (to C.L.T.), and C.T.S. was supported by NIH training grant HL07553.

REFERENCES


17. Lukomski, S., K. Nakashima, I. Abdil, V. J. Cipriano, R. M. Ireland, S. D. Heinz, C. G. Adams, and J. Engel. 2003. Circular-dichroism and binding of peptide ligands and MAbs to their respective receptors and antigens located in the basal layer of the exosporium (4; D. D. Williams and C. L. Turnbough, Jr., unpublished data). Presently, several laboratories are actively investigating possible roles for BclA and the entire exosporium in spore virulence. Although such roles remain to be defined, it seems likely that the properties of BclA described in this study contribute to the fate of \textit{B. anthracis} spores whether they are lying dormant in the soil or initiating an infection within tissues of a mammalian host.