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Light Chain of Botulinum A Neurotoxin Expressed as an Inclusion Body from a Synthetic Gene Is Catalytically and Functionally Active

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Botulinum neurotoxins, the most potent of all toxins, induce lethal neuromuscular paralysis by inhibiting exocytosis at the neuromuscular junction. The light chains (LC) of these dichain neurotoxins are a new class of zinc-endopeptidases that specifically cleave the synaptosomal proteins, SNAP-25, VAMP, or syntaxin at discrete sites. To facilitate the structural and functional characterization of these unique endopeptidases, we constructed a synthetic gene for the LC of the botulinum neurotoxin serotype A (BoNT/A), overexpressed it in *Escherichia coli*, and purified the gene product from inclusion bodies. Our procedure can provide 1.1 g of the LC from 1 L of culture. The LC product was stable in solution at 4°C for at least 6 months. This rBoNT/A LC was proteolytically active, specifically cleaving the Glu–Arg bond in a 17-residue synthetic peptide of SNAP-25, the reported cleavage site of BoNT/A. Its calculated catalytic efficiency k_{cat}/K_m was higher than that reported for the native BoNT/A dichain. Treating the rBoNT/A LC with mercuric compounds completely abolished its activity, most probably by modifying the cysteine-164 residue located in the vicinity of the active site. About 70% activity of the LC was restored by adding Zn^{2+} to a Zn^{2+}-free, apo-LC preparation. The LC was nontoxic to mice and failed to elicit neutralizing epitope(s) when the animals were vaccinated with this protein. In addition, injecting rBoNT/A LC into sea urchin eggs inhibited exocytosis-dependent plasma membrane resealing. For the first time, results of our study make available a large amount of the biologically active toxin fragment in a soluble and stable form.

KEY WORDS: Botulinum neurotoxin; zinc-endopeptidase; light chain; apo-light chain; exocytosis; inclusion body; synthetic gene.

1. INTRODUCTION

Botulinum and tetanus neurotoxins are a new class of zinc-endopeptidases that act selectively at discrete sites on three synaptosomal proteins of the neuroexocytotic apparatus (see Montecucco and Schiavo, 1995, and Schiavo, 1995, for review). These neurotoxins are the most potent of all the known toxins. The botulinum neurotoxins (BoNT)[3], designated A–G, produced by seven immunologically distinct strains of *Clostridium botulinum* cause death by flaccid muscle paralysis at the neuromuscular junction. Extreme toxicity of these toxins and their lability in purified preparations have limited any detailed characterization.

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[3] Abbreviations: BoNT, Botulinum neurotoxin; BoNT/A, botulinum neurotoxin serotype A; LC, light chain; HC, heavy chain; H_n, N-terminal domain of the heavy chain; H_c, C-terminal domain of the heavy chain; SNAP-25, synaptosomal associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; SNARE, soluble NSF attachment protein receptor; IPTG, isopropyl β-D-thiogalactopyranoside; GuHCl, guanidine hydrochloride; EDTA, ethylene diamine tetraacetate; PMSF, phenylmethanesulfonyl fluoride; ABTS, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; SKL, sarkosyl; pCMB, *p*-chloromercuribenzoate; MALDI, matrix-assisted laser desorption-ionization: ELISA, enzyme-linked immunosorbent assay.

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These neurotoxins are expressed as 150-kDa single polypeptides (termed dichains) containing a disulfide bond between the 50-kDa N-terminal light chain (LC) and the 100-kDa C-terminal heavy chain (HC). A post-translational tryptic cleavage generates the two chains that are still connected by the disulfide bond. The LC contains the toxic, zinc-endopeptidase catalytic domain. The 100-kDa HC can further be proteolyzed into a 50-kDa N-terminal membrane-spanning domain (H_n) and a 50-kDa C-terminal receptor-binding domain (H_c).

With three functional domains, the mechanism of action of these neurotoxins is multiphasic: (1) The H_c domain plays a role in binding the toxins to specific receptors located exclusively on the peripheral cholinergic nerve endings (Black and Dolly, 1986). (2) The H_n domain is believed to participate in a receptor-mediated endocytotic pore formation in an acidic environment, allowing translocation of the catalytic LC into the cytosol. Reducing the disulfide bond connecting the LC with the H_n upon exposure to the cytosol or within the acidic endosome (Montal *et al.,* 1992) releases the catalytic LC into the cytosol. (3) The LC then cleaves at specific sites of one of the three different SNARE proteins, synaptobrevin, syntaxin, or SNAP-25 (Blasi *et al.*, 1993; Schiavo *et al.,* 1993, 1994; Shone *et al.,* 1993; Foran *et al.,* 1996). These proteins are essential for synaptic vesicle fusion in exocytosis. Their proteolysis inhibits exocytosis and blocks acetylcholine secretion, leading ultimately to muscular paralysis. The LC itself is nontoxic as it cannot translocate through the cholinergic nerve ending into the cytosol. However, in digitonin-permeabilized chromaffin cells, the LC inhibits exocytosis (Bittner *et al.,* 1989), and direct microinjection of the LC into the cytosol results in blockage of membrane exocytosis (Bittner *et al.,* 1989; Bi *et al.,* 1995).

The LC of all known clostridial neurotoxins contain the sequence HExxH that is characteristic of zinc-endoproteinases (Thompson *et al.,* 1990). The essential role of zinc on the structure and catalysis of the neurotoxins is established (Fu *et al.,* 1998). A unique feature of the neurotoxins' protease activity is their substrate requirement. Short peptides encompassing only the cleavage sites are not hydrolyzed (Foran *et al.,* 1994; Shone and Roberts, 1994). A specific secondary and/or tertiary structure of the substrate is most probably recognized (Washbourne *et al.,* 1997; Lebeda and Olson, 1994; Rossetto *et al.,* 1994) rather than a primary structure alone, as is the case with most other proteases. Most importantly, their identified natural substrates are proteins involved in the fundamental process of exocytosis (Blasi *et al.,* 1993; Schiavo *et al.,* 1993, 1994; Shone *et al.,* 1993; Foran *et al.,* 1996). Light chain also is the target of an intensive effort to design drugs, inhibitors, and vaccines. A detailed understanding of its structure and function is thus very important.

Commercially available LC is prepared by separating it from the dichain toxins under denaturing conditions. These preparations therefore retain some contaminating toxicity of the dichain, have low solubility, and often begin to proteolytically degrade and start losing activity within hours of storage in solution. The LC of BoNT/A has been expressed as a maltose-binding protein, and was purified to 0.5 mg from a 1-L culture (Zhou *et al.,* 1995). The poor expression in *E. coli* of this cloned product is probably due to rare codons of the clostridial DNA (Winkler and Wood, 1988). The lack of a method for obtaining large amounts[4] of a stable and active LC is a major hurdle for their detailed structural and functional characterization. In this paper, we describe the construction and overexpression of a synthetic gene for the nontoxic LC of BoNT/A in *E. coli.* The high level of expression obtained allowed us to obtain and purify the LC in gram quantities from 1 L of culture and extensively characterize it. Our preparation of the rBoNT/A LC was highly soluble, stable at 4°C for at least 6 months, and had the expected enzymatic and functional properties. For the first time, we also tentatively identified a cysteine residue in the vicinity of the active site which, when modified by mercuric compounds, led to complete loss of enzymatic activity.

2. MATERIALS AND METHODS

2.1. Chemicals, Buffers, and Reagents

Buffer T (20 mM Tris-HCl, pH 9.2) and buffer G (50 mM sodium glycine, pH 9.0) were used as indicated. SKL (sodium N-lauryl sarcosine or sarkosyl) was from Sigma. Highly purified (>95%) full-length BoNT/A was purchased from List Biologicals (Campbell, CA). Rabbit polyclonal antibodies against a 16-residue N-terminal sequence (PFVNKQFNYKDPVNGV) of the BONT/A LC were produced and affinity purified by Research Genetics (Huntsville, AL). Peroxidase-coupled goat anti-rabbit and anti-mouse IgG (H + L) and ABTS substrate were from Kirkegaard Perry Laboratories (Gaithersburg, MD). Oligonucleotides, designed for *E. coli* codon usage (Andersson and Kurland, 1990) and ranging in size from 70 to 100 nucleotides, were synthesized by Macromolecular Resources (Fort Collins, CO).

[4] When this manuscript was being prepared, a report appeared on the expression and purification of rBoNT/A with a yield of 20 mg from 1-L culture (Li and Singh, 1999).

2.2. Construction of the Synthetic Gene and Expression of the rBoNT/A LC

The gene encoding the enzymatic LC domain of BoNT/A was assembled from three segments, a 335-base pair (bp) *Sal* I–*Sph* I fragment, a 600-bp *Sph* I–*Kpn* I fragment, and a 460-bp *Kpn* I–*EcoR* I fragment. To construct the first segment, six oligonucleotide pairs were annealed, ligated, and, after PCR amplification, inserted into pGEM3Zf at *Sal* I–*Sph* I restriction enzyme sites. The second segment was built by annealing and ligating eight oligonucleotide pairs, followed by its amplification and insertion into the *Sph* I and *Kpn* I sites of pGEM3Zf. The final segment was constructed by annealing and ligating six oligonucleotide pairs, followed by its amplification and insertion into the *Kpn* I–*EcoR* I sites of pGEM3Zf. Nucleotide sequencing of gene fragments in pGEM3Zf was performed to identify clones in each group with minimal misincorporations. *In vitro* mutagenesis was performed to correct the misincorporations in the BoNT/A LC minigene fragments. Directional gene assembly via 600-bp and 460-bp fragments in pGEM3Zf was followed by the insertion of the 335-bp fragment.

In the design of the synthetic gene, the 5′ oligonucleotide for amplifying the gene's 5′ terminus consisted of an anchored *Sal* I site followed by an *EcoR* I site and an *Nco* I site to facilitate directional subcloning into the *E. coli* expression vector, pET24d. The 3′ oligonucleotide contained a hexahistidine tag with a thrombin protease cleavage site for creating a carboxyl-terminal removable histidine tag. The 3′ end also included the restriction enzyme sites for *BamH* I and *EcoR* I.

The full-length gene was excised from pGEM3Zf with a *Nco* I–*EcoR* I and subcloned into a similarly digested pET24d vector. The resulting ligated construct was used to transform *E. coli* BL21(DE3) cells. Two clones were assayed for their ability to express rBoNTA LC. Single colonies were inoculated into 5 ml of Luria broth (LB) containing 50 μg/ml of kanamycin and grown overnight at 37°C. The overnight cultures (500 μl) were used to inoculate 50 ml of LB containing 50 μg/ml of kanamycin. When the cultures reached OD_{600} of 0.8, induction was initiated by addition of isopropyl-β-D-thiogalactoside (IPTG) (final concentration, 1.0 mM). The cultures were induced for 2 hr at 37°C, harvested, and analyzed for expressed products on SDS–PAGE.

2.3. Fermentation

A frozen stock seed culture of recombinant *E. coli* harboring the synthetic gene encoding the LC of BoNT/A was grown at 37°C to an OD_{600} of 2.682 in a shake flask containing 100 ml of the following defined medium: casamino acids (1.4 g/L); yeast extract (2 g/L); $(NH_4)_2SO_4$ (1.85 g/L); K_2HPO_4 (30 g/L); $MgSO_4 \cdot 7H_2O$ (2 g/L); thiamine·HCl (0.015 g/L); glucose (18.1 g/L); trace elements solution (3 ml/L) consisting of $FeCl_3 \cdot 6H_2O$, 27 g; $ZnCl_2 \cdot 4H_2O$, 1.3 g, $CoCl_2 \cdot H_2O$, 2 g; $Na_2Mo_4 \cdot 2H_2O$, 2 g; $CaCl_2 \cdot 2H_2O$, 1 g; $CuCl_2 \cdot 2H_2O$, 1 g; H_3BO_3, 0.5 g; distilled H_2O, 1000 ml; and HCl, 100 ml. In addition, 0.0156 g/L of ZnCl was added to trace minerals to make the concentration of Zn five times greater in the shake flask and fermentor. Kanamycin (50 μg/L) was added as an antibiotic. The shake flask culture was used to inoculate a 5-L BioFlo III fermentor (New Brunswick Scientific, Edison, NJ) containing 4.3 L of the medium described above. Later in the growth (5.5 hr), 14.1 g/L of casamino acids was added and a glucose feed was initiated to maintain a glucose concentration of 1 g/L. Growth continued for 8 hr until an OD_{600} of 49.9 was reached. Cell induction was then initiated at this time by adding IPTG (final concentration, 1.5 mM). Induction continued for 4 hr after adding IPTG, and cells (OD_{600} of 112.62) were harvested by centrifugation (Beckman, Palo Alto, CA) at 7000 rpm for 15 min at 4°C. Cells were washed with cold 0.9% saline and centrifuged at 7000 rpm for 15 min and frozen at–70°C. Wet cell yield was 58 g/L.

2.4. Extraction and Purification of Light Chain as Inclusion Bodies

In a typical preparation, 12 g of *E. coli* cells was suspended in a total volume of 30 ml of buffer T containing 5 mM $MgCl_2$, 1.5 mM PMSF, 10 mM β-mercaptoethanol, and 2 mg of DNAse. The cell suspension was subjected to 10 cycles of 2-min sonication (at 60% power in a Fisher Model 300 Sonic Dismembrator) and 2-min cooling on ice. After centrifugation for 15 min at 10,000 × *g*, the supernatant was discarded. The pellet was suspended in 30 ml of the above buffer. The cycle of sonication and centrifugation was repeated five more times; $MgCl_2$ and DNAse were omitted from the buffer during the last two cycles. The resulting pellet contained the rBoNT/A LC that appeared ~70% pure by SDS PAGE (Fig. 2). The pellet was stored at 4°C as a white suspension in 15 ml of buffer T containing 1.5 mM PMSF and 10 mM β-mercaptoethanol.

2.5. Solubilization of the Inclusion Bodies to Obtain Active rBoNT/A LC

In a typical experiment, 0.75 ml of the white rBoNT/A LC suspension (from an equivalent of 600 mg of wet cells) was centrifuged in a 2-ml Eppendorf tube and

the supernatant was discarded. The pellet was suspended by mild sonication in 0.9 ml of 50 mM Tris-HCl, pH 9. A 20% solution (0.9 ml) of SKL in water was added to the suspension at room temperature and was mixed by inversion several times. Within 2 min, the pellet became completely soluble. Any remaining turbidity was cleared by further diluting with 50 mM Tris-HCl, pH 9.0, or was removed by centrifugation. The SKL-solubilized LC was dialyzed against 200 volumes of buffer G containing 1 mM DTT with one to two daily changes at 4°C for 1 week. The yield of the soluble rBoNT/A LC was 12 mg (3.9 mg/ml), which was stored in a glass tube at 4°C.

2.6. Preparation of Apo-rBoNT/A LC

One milliliter of rBoNT/A LC (2.73 mg) was dialyzed overnight against 250 ml of buffer G containing 5 mM EDTA and 1 mM DTT. EDTA was removed by further dialysis for 60 hr against three changes of 250 ml of buffer G containing 1 mM DTT.

2.7. Assay of Proteolytic Activity of BoNT/A LC

The assay is based on HPLC separation and measurement of the nicked products from a 17-residue C-terminal peptide of SNAP-25 (Fig. 5), corresponding to residues 187–203, which is the minimum length required for BoNT/A proteolytic activity (Schmidt and Bostian, 1995, 1997). Unless otherwise noted, a 0.03-ml assay mixture containing 0.8–1.0 mM substrate, 0.25 mM $ZnCl_2$, 5.0 mM DTT, 50 mM Na-HEPES buffer (pH 7.4), and BONT/A LC was incubated at 37°C for 15–180 min. The amounts of uncleaved substrate and the products were measured after separation by reverse-phase HPLC (Waters) on a Hi-Pore C18 column, 0.45 × 25 cm (Bio-Rad Laboratories, Hercules, CA) with the Millennium software (Waters) package. Solvent A was 0.1% TFA and solvent B was 70% acetonitrile/0.1% TFA. The flow rate was 1.0 ml/min at 25°C. After the column was eqilibrated with 10% B, the sample was injected, and the column was held at 10% B for 2.5 min. A linear gradient to 36% B over 21 min was followed by 100% B for 6 min. Kinetic parameters for the synthetic substrate were calculated from Lineweaver–Burk plots of activity with peptide concentrations from 0.26 to 1.7 mM.

2.8. Vaccination of Animals

Purified rBoNTA LC was tested for its ability to elicit protective immunity in Cr1:CD-1 (ICR) male mice[5] (Charles River) weighing 16–22 g. Two concentrations of recombinant LC (5 and 15 μg) with and without adsorption to a 0.2% Alhydrogel (Superfos Biosector, Kvisgaard, Denmark) were administered in 0.9% saline in a total volume of 100 μl. Groups of 10 mice including a naive control (saline alone) received three doses of LC at 0, 2, and 4 weeks. Mice were bled from the retroorbital sinus 12 days postvaccination and their antibodies assayed for titers to toxin. Animals were challenged with native BoNT/A dichain toxin 15 days postvaccination.

2.9. ELISA

Highly purified (>95%) BoNT/A toxin was diluted to 2 μg/ml in phosphate-buffered saline (PBS), pH 7.4 (Sigma Chemical Co., St Louis, MO) and was dispensed (100 μl/well) into microtiter plates (Immulon 2, Dynatech Laboratories, Chantilly, VA). The plates were incubated overnight in a humidity box at 40°C. Five percent skim milk (Difco, Detroit, MI) in PBS with 0.01% Thimerosal® was used to block nonspecific binding and as an antibody diluent. The plates were washed with PBS plus 0.1% Tween 20 between each step. Mouse sera were initially diluted 1:100 and then diluted fourfold for a total of eight dilutions (1:100 to 1:1,600,000). Diluted sera were added in duplicate to toxin-coated wells (100 μl/well). The secondary antibody was horseradish peroxidase-conjugated, goat anti-mouse IgG diluted 1:1000. The primary and secondary antibodies were incubated 90 and 60 min, respectively at 37°C. ABTS substrate (100 μl/well) was added as the color developer. The plates were incubated at room temperature for 30 min. The absorbance was measured with a microplate reader at 405 nm. A mouse monoclonal antibody, 5BA2.3, was used as the positive control in each assay; naive mouse serum was added as a negative control in each assay. The titer was defined as the geometric mean of the ELISA titer to BoNT/A toxin.

[5] The animal room was maintained at 21±2°C with a relative humidity 30–70%, a 12/12-hr light/dark cycle with no twilight, and 10–15 air changes/hour. Mice were housed in solid-bottom, polycarbonate Micro-Isolator™ cages (Lab Products, Inc., Seaford, DE) with paper chip bedding (Alpha-Dri™, Shepherd Specialty Papers, Inc., Kalamazoo, MI) and provided food (Harlan Teklad diet No. 7022, NIH-07) and water *ad libitum.* All procedures were reviewed and approved by the Institutional Animal Care and Use Committee and performed in an AAALAC International-accredited facility in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals,* 1996 (National Academy Press, National Academy of Sciences, Washington, D.C.).

2.10. Exocytosis Experiments

Plasma membrane resealing after micropuncture with a glass pipette requires calcium-regulated exocytosis (Bi *et al.*, 1995). This exocytosis is dependent on docking proteins (the SNARE complex) that are sensitive to proteolysis by the clostridial neurotoxins (Steinhardt *et al.*, 1994). Sea urchin (*Lytechinus pictus*) eggs were used to test the biological activity of the rBoNT/A LC. The microinjection medium contained 19 volumes of the rBoNT/A LC (3.7 mg/ml) in 45 mM potassium aspartate, 5 mM HEPES, pH 8.1, and one volume of 55 mM fura-2 in 100 mM KCl and 10 mM HEPES, pH 7.1. Injection levels were 5–10% of egg volume. The plasma membrane resealing after micropuncture with a glass pipette was monitored by recording the emission from fura-2 upon excitation at 358 nm (the calcium-insensitive wavelength).

2.11. Other Analytical Methods

Protein concentration was determined by BCA assay (Pierce) with bovine serum albumin (BSA) as a standard. Reducing SDS–PAGE with 10% tricine-gels (Novex) was according to Laemli (1970). The gels were stained with Coomassie brilliant blue. Western blots were prepared by using a primary polyclonal antibody against a 16-residue N-terminal sequence of BoNT/A LC and a peroxidase-coupled goat anti-rabbit IgG (H+L) as the secondary antibody. Absorption spectrum at 25°C was recorded in a Hewlett-Packard 8452 diode array spectrophotometer. The N-terminal amino acid sequence of the BoNT/A LC was determined by Edman degradation in a Applied Biosystems Procise Sequencer in the 0- to 20-pmol detection range. Molecular mass was determined by MALDI-MS in a PE Biosystems Voyager DE instrument. Sinapinic acid was used as the matrix and the sample was spotted on a stainless steel plate that was not washed with water or TFA. Other conditions in the experiment were accelerating voltage 25,000 V, guide wire voltage 0.3%, and laser 2500.

3. RESULTS

3.1. Synthetic Gene Construction and Expression of BoNT/A Light Chain

A synthetic gene encoding rBoNTA LC was designed with *E. coli* codon usage, constructed, and expressed in *E. coli.* The natural gene sequence from *C. botulinum* type A NTCC 2916 (Thompson *et al.*, 1990) was used as the template for our synthetic LC sequence. At the 5′ end of the gene, we employed an *Nco* I restriction enzyme site as a cloning site and palindrome to provide an initiation codon. The use of this *Nco* I site necessitated the use of a filler codon (GTT) between the Met initiation codon (ATG) and the codon (CAG) specifying the first amino acid residue in the LC (i.e., Q). This resulted in the introduction of one extra amino acid, Val, as the N-terminal residue (after the initiating Met). This extra and new amino acid, however, did not interfere with expression or activity (see later). The length of the LC (448 residues) to be expressed was chosen from the sequence of amino acids around the nicking site (DasGupta and Dekleva, 1990) (Fig. 1). At the C-terminal end (i.e., DKGYNK), we incorporated a hexa-His tag for affinity purification and a thrombin cleavage site (LVPRGS) for removing the hexa-His tag. The expressed protein therefore contained a total of 461 (1 + 448 + 6 + 6) residues (Fig. 1). The synthetic gene thus constructed in pET24d vector was highly and efficiently expressed in *E. coli*, accounting for about 25% of the total protein (Fig. 2).

3.2. Purification of the rBoNT/A LC

The expressed LC appeared exclusively in the insoluble pellet fraction (Fig. 2). Including $MgCl_2$ and DNAse in the cell suspension ensured a clean separation of the pellet from the supernatant after sonication and centrifugation. The white suspension of the purified BoNT/A LC migrated as a 52-kDa band and appeared to be ~70% pure on SDS–PAGE (Fig. 2A), as determined by densitometric analysis. Minor contaminant bands with ~100-kDa, 37–40 kDa, and ~25 kDa also reacted with the antibody in the Western blot (Fig. 2B). While fragments smaller than 50 kDa may have arisen from proteolysis of the LC (DasGupta and Foley, 1989), the origin of the 100-kDa species in the reducing SDS–PAGE gels is not clear since the species also reacts with the affinity-purified antibodies against a small sequence of the LC. Molecular mass determination by MALDI-MS gave 52.774 (±50) kDa as the predominant species along with minor species of 106.028 (±100) kDa and 25.00 (±25) kDa. Amino acid sequence determination of the LC identified V-Q-F-V-N-K-Q as the amino-terminal sequence, as expected for the constructed gene (Fig. 1) and identical (with the exception of the penultimate valine) to that of the published sequence of BoNT/A (Thompson *et al.*, 1990).

The purified inclusion bodies were solubilized in 10% SKL and the SKL was removed by dialysis against buffer G containing 1 mM DTT (see Section 2). The use of a 10% SKL solution ensured solubilization within

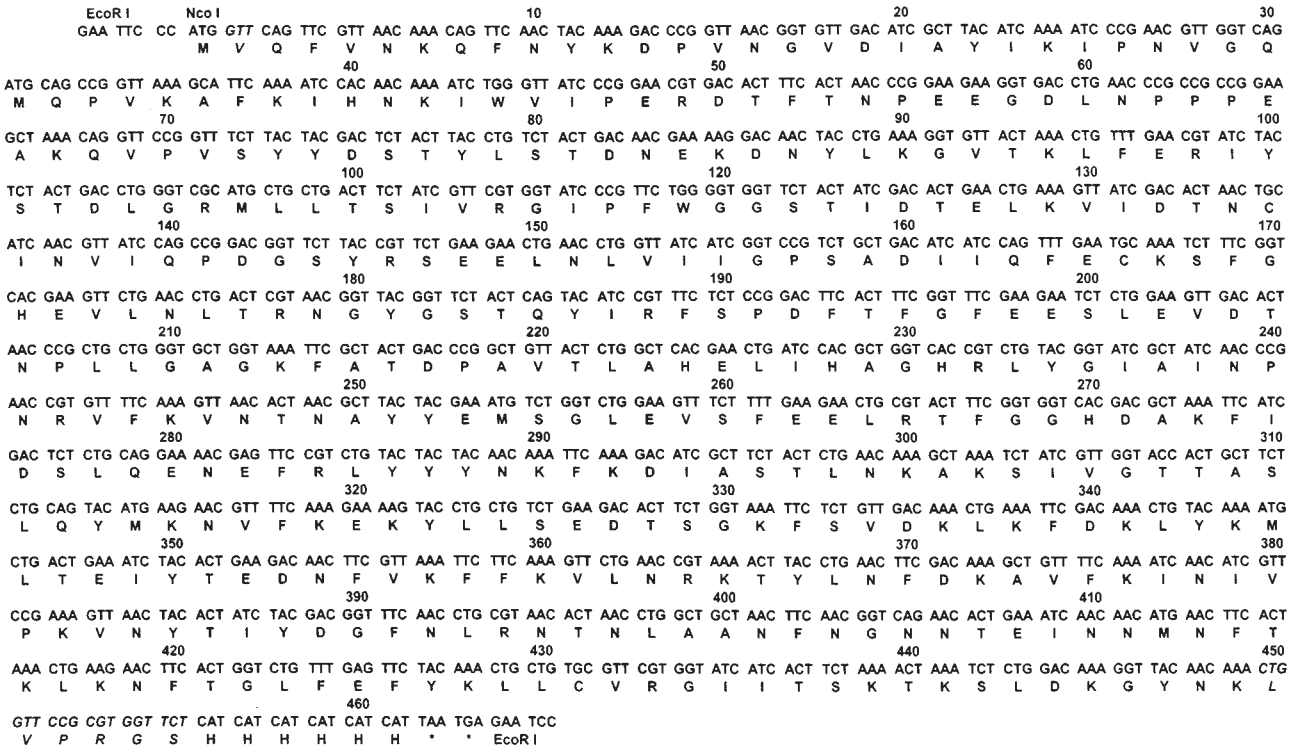

Fig. 1. Nucleotide sequence of rBoNT/A LC and the corresponding amino acid sequence. The codon in *italics* (i.e., encoding the penultimate val residue) at the 5′ end of the gene was introduced to create and maintain the *Nco* I restriction enzyme site. Codons in *italics* (i.e., encoding LVPRGS) at the 3′ end of the gene encode a thrombin protease cleavage site for removing the His tag after purification.

2 min of incubation, and the LC solution was immediately subjected to extensive dialysis to remove the detergent. Starting with an equivalent of 600 mg of the wet *E. coli* cells, we obtained 12 mg of the soluble LC corresponding to 20 mg LC per gram of wet cell. This corresponds to a yield of 1.16 g of the pure protein per liter of cell culture.

3.3. Properties of the Purified BoNT/A LC

The UV-visible absorption spectrum (Fig. 3) shows the rBoNT/A LC with a single maximum at 278 nm as a simple protein. Although a number of minor bands were observed in the SDS–PAGE gel (Fig. 2), absence of any other absorbance bands in the UV–visible range suggests the absence of any nonmetal cofactor in the preparation. The LC was expressed as a C-terminally His-tagged protein. In the presence of 6 M GuHCl, the rBoNT/A LC was bound to Ni-resin and was eluted with immidazole-containing buffers as a more purified form (not shown). Without GuHCl, the rBoNT/A LC did not bind to Ni-resin. This result suggests that the LC retained the His-tag after expression and purification, but in the absence of GuHCl, the His-tag was not exposed to solvent to chelate with the Ni-resin. Because the rBoNT/A LC had catalytic properties comparable to those of the dichain (see below), we did not attempt to remove the His-tag from the purified protein.

The purified LC was stable for at least 6 months when stored at 4°C in buffer G containing 1 mM DTT (Fig. 4A). During this period, the protein remained fully soluble, did not show any degradation as analyzed by SDS–PAGE, and retained its initial catalytic activity. A LC preparation obtained by prolonged solubilization in 0.5% SKL at room temperature, however, precipitated after 3 months of storage at 4°C and lost most of its initial catalytic activity. The LC (1 mg/ml of 50 mM Na-phosphate) precipitated from solution below pH 8 either at 4°C or at 25°C. Thermal stability of the LC (3.74 mg/ml of buffer G containing 1 mM DTT and 50 μM $ZnCl_2$) was investigated by incubating aliquots for 5 min at various temperatures. After cooling on ice for 45 min, the catalytic activities in the supernatants were measured. The midpoint of thermal unfolding T_m as measured by activity was 43°C (Fig. 4B). At room temperature, increasing concentration of $MgCl_2$ also precipitated the LC

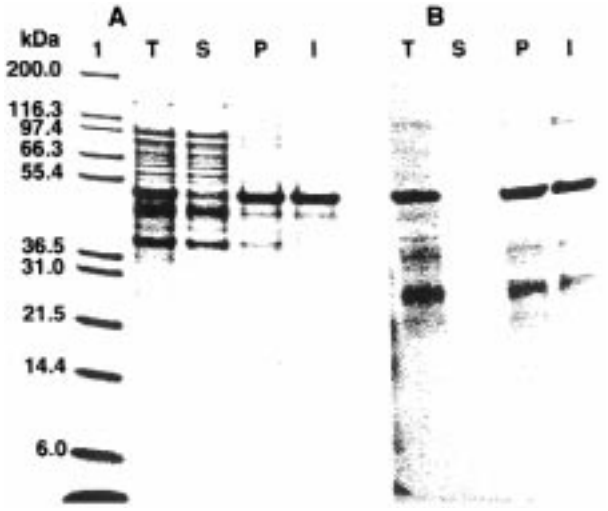

Fig. 2. SDS–PAGE followed by Coomassie stain (A) and Western blot (B) of crude and purified BoNT/A LC expressed in *E. coli* containing the synthetic gene for BoNT/A LC in a multicopy plasmid pET24. Total cellular protein (T), soluble supernatant (S), insoluble pellet (P), and purified inclusion bodies (I) were prepared as described in Section 2. Lane 1 shows Novex wide-range molecular-mass markers (0.8–3.0 μg/band). The sarkosyl solubilized inclusion bodies of the LC had the same electrophoretic behavior as (I) (not shown). About 20 μg of protein was applied per lane. Western blot used affinity-purified rabbit polyclonal antibodies against a 16-residue N-terminal sequence of the BoNT/A LC as the primary antibody and a peroxidase-coupled goat anti-rabbit IgG (H+L) as the secondary antibody. Bands were visualized by a chromogenic substrate.

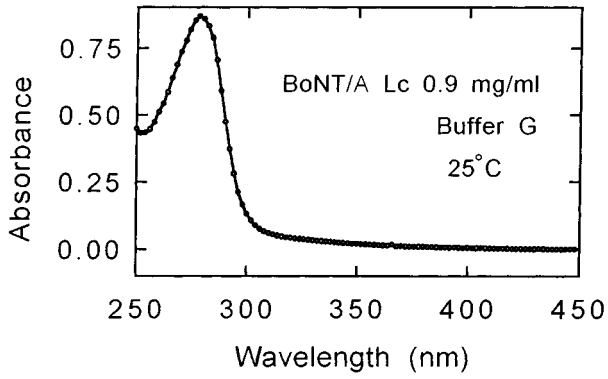

Fig. 3. UV-visible absorption spectrum of the rBoNT/A LC.

from solution: at 6 mM $MgCl_2$, >80% of the LC precipitated (not shown).

3.4. Biological Effects of the rBoNT/A LC

LC prepared from dichain BoNTs always had residual toxicity due to some contaminating dichain forms

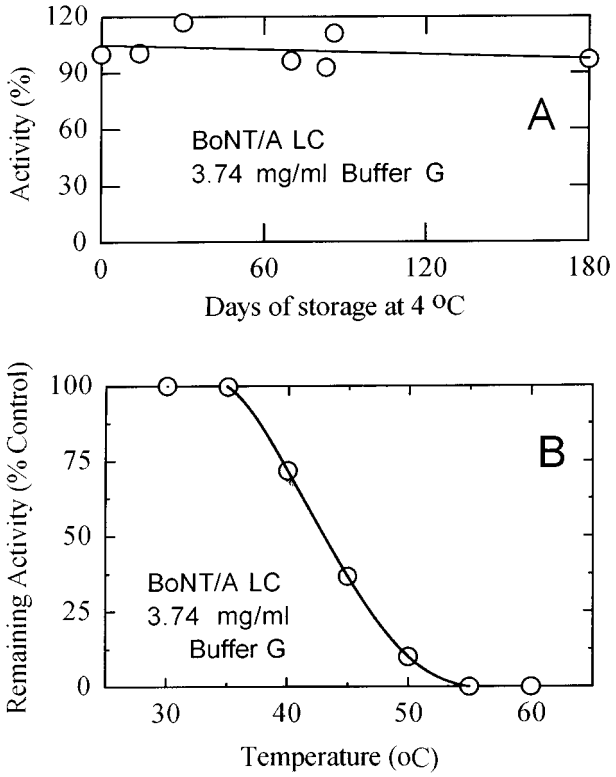

Fig. 4. Long-term stability at 4°C (A) and thermal stability (B) of the rBoNT/A LC. (A) Aliquots of the LC from one single preparation were assayed at the indicated times; (B) 50-μl aliquots of the LC in buffer G containing 1 mM DTT and 50 μM $ZnCl_2$ were taken in Eppendorf tubes and heated for 5 min at the indicated temperatures. After cooling on ice for 60 min, the supernatants were assayed for proteolytic activity.

(Maisey *et al.*, 1988). To demonstrate and confirm that the rBoNT/A LC was nontoxic, we injected 5–15 μg of the LC per mouse, a dose that was 15,000–45,000 times higher than an equivalent lethal dose of the BoNT/A dichain. Table I shows that all the mice survived three successive injections. All of their antisera had high titers against BoNT/A, but these antibodies failed to protect the animals upon subsequent challenge with relatively low doses (10^2 LD_{50}) of the toxic BoNT/A dichain. Even when the ELISA titers were boosted 20-fold by using the aluminum hydroxide adjuvant, the animals were not immune to modest levels of BoNT/A challenge (Table I). Comparable vaccination with BoNT/A H_c protected animals from challenge with as high as 10^6 LD_{50} (Smith, 1998). Our results clearly demonstrate that the rBoNT/A LC was nontoxic to the animals and confirms earlier observations that LC does not possess any neutralizing epitope(s) (Chen *et al.*, 1997; Dertzbaugh and West, 1996).

Although the LC by itself is nontoxic, in digitonin-permeabilized chromaffin cells (Bittner *et al.*, 1989) and

Table I. Survival of Mice after Vaccination with the rBoNT/A LC and Subsequent Challenge by BoNT/A Dichain

Dose[a] (μg/mouse)	ELISA titer[b]	Survival at given BoNT/A dichain challenge[c]	
		10^2 LD_{50}	10^3 LD_{50}
0[d]	<100	0/5	0/5
5[d]	18,000	0/10	0/10
15[d]	63,100	0/10	0/10
0[e]	<100	0/5	0/5
5[e]	985	0/10	0/10
15[e]	2800	0/10	0/10

[a] Each mouse was injected at 0, 2, and 4 weeks with the indicated dose.
[b] Titer is the reciprocal of the highest dilution with an A_{405} of greater than 0.2 after background correction. Values are geometric means of the titers of the sera obtained 12 days after final injection.
[c] LD_{50} is an empirically determined dose of the neurotoxin necessary to cause lethality in 50% of a population of mice.
[d] The LC was adsorbed on Alhydrogel and used as the immunogen.
[e] The LC in saline was used as the immunogen.

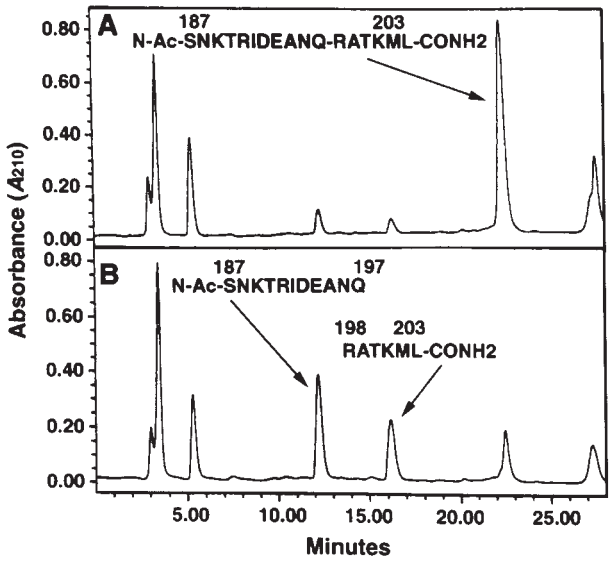

Fig. 5. Proteolysis of the synthetic peptide substrate by the rBoNT/A LC. The peptide (1.1 mM) was incubated for 5 min (**A**) or 200 min (**B**) with the rBoNT/A LC. The reaction products were analyzed by reverse-phase HPLC. The first three peaks represent the solvent front (<4 min) and reduced DTT (5.2 min) in the reaction mixture. Sequence of the substrate and the sequences of the products are shown in panels A and B, respectively. The numbers above the sequences represent the residue numbers corresponding to the sequence of SNAP-25. The product peaks (not labeled in Panel A) were identified by sequence determination by MS-MS.

direct microinjection into the cytosol of sea urchin eggs (Bi *et al.*, 1995; Steinhardt *et al.*, 1994), it blocks membrane exocytosis. To demonstrate that our rBoNT/A LC preparation retained this property of inhibiting membrane exocytosis, sea urchin eggs were microinjected with the LC. Eggs of the sea urchin, *Lytechinus pictus*, are an excellent model system for the study of exocytosis. Unfertilized eggs have a layer of vesicles, the cortical granules, docked at the plasma membrane. The SNARE complexes of docked vesicles are inaccessible to the BoNTs. Thus, plasma membrane resealing of the unfertilized sea urchin egg is unaffected by microinjection with botulinum toxins A, B, and C1 (Bi *et al.*, 1995; Steinhardt *et al.*, 1994). Fertilization triggers exocytosis of the cortical granuoles. After fertilization, the vesicles available for exocytosis are largely undocked and the docking proteins of undocked vesicles are susceptible to proteolysis by injected clostridial neurotoxins.

For fertilized eggs injected with rBoNT/A LC, about 100 min at 20°C was required to inhibit plasma membrane resealing after mechanical wounding with a glass micropipet. Eggs that successfully resealed showed a transient dye loss for about 1–2 min after micropuncture. Eggs that failed to reseal continuously lost dye and lost control of intracellular free calcium, leading to cell death. Five of five fertilized eggs wounded between 36 and 70 min after injection with the rBoNT/A LC resealed successfully, as did five of five unfertilized injected eggs. Six of six fertilized eggs wounded between 106 and 145 min after injection failed to reseal, indicating that the recombinant light chain actively inhibited exocytosis. Thus, the rBoNT/A LC had a similar effect as BoNT/B in inhibiting membrane exocytosis and resealing of plasma membrane of sea urchin eggs (Steinhardt *et al.*, 1994).

3.5. Catalytic Activity of the LC

The BoNT/A LC is zinc-endopeptidase specific for cleaving the peptide bond between residues 197 (Glu) and 198 (Arg) of SNAP-25. Incubating the 17-mer synthetic peptide representing residues 187–203 of SNAP-25 with the LC at 37°C for 5–200 min generated only two peptides (Fig. 5). That no other peptide fragments were generated by this prolonged incubation proves that the contaminants present in the LC preparation were devoid of any proteolytic activity. Incubating the LC with BSA also failed to produce any proteolytic fragment (not shown). In contrast to the BoNT/A dichain, whose activity is greatly enhanced by BSA (Schmidt and Bostian, 1997), the rate of cleavage of the synthetic peptide substrate was unaffected by the presence of BSA.

Proteolytic activity of the purified rBoNT/A LC linearly increased with the increasing amount of the LC

in the reaction mixture. The time course of activity (at 0.8–1.0 mM substrate concentration), however, was not linear, but progressively declined, possibly due to a high K_m for the substrate peptide (see below). Therefore, routine assays depended on initial activities representing <30% substrate conversion.

3.6. Optimum pH, Salts, and Buffers

An optimum pH of 7.2 for the proteolysis of the synthetic substrate by the rBoNT/A LC was determined by assaying in three different buffer systems (0.1 M) ranging in pH from 5.0 to 9.0 (Fig. 6). For comparison, the optimum pH values of BoNT/B and and tetanus neurotoxin, two members of the clostridial neurotoxin family, are 6.5–7.0, and 6.5–7.5, respectively (Foran *et al.,* 1994). Tris-HCl appeared to have an inhibitory effect on proteolysis, presumably due to chelation with the zinc at the active site. The activity at pH 7.4 was 25% higher in a 50 mM HEPES buffer than in 100 mM HEPES. Adding 50 mM NaCl, KCl, or $NaPO_4$ (pH 7.4) to the standard reaction mixture reduced activity 40–50%. Thus, high salt concentrations inhibited the proteolytic reaction.

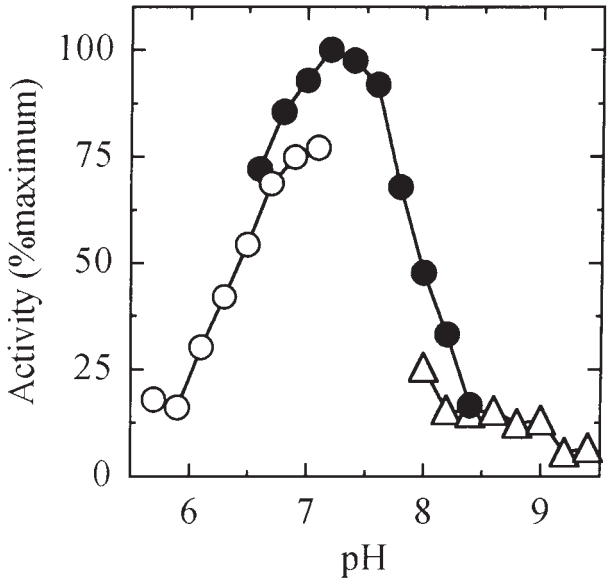

Fig. 6. Effect of pH on the endopeptidase activity of the rBoNT/A LC. Activities were measured at various pH of 0.1 M buffers: MES (-○-), HEPES (-●-), and tris-HCl (-△-) containing 0.9 mM substrate peptide. Maximum activity (100%) was 334 nmol/min/mg LC.

3.7. Effect of Metals and Thiol Reagents on Activity

BoNT/A LC is a zinc-endopeptidase. Activity of the rBoNT/A LC was completely inhibited by including the metal chelator EDTA (1 mM) in the reaction mixture (Table II). Adding low concentrations of $ZnCl_2$ (1–50 μM) in the assay mixture slightly stimulated the activity (5–10%), and higher concentrations of $ZnCl_2$ inhibited the activity (Fig. 7). The results suggest that the active site should be almost saturated with Zn^{2+} for optimum activity. The metal was tightly bound to the active site of the LC, as the extraction, purification, or dialysis buffers were devoid of Zn^{2+}. Like Zn^{2+}, other divalent metal ions, notably, $MnCl_2$ and $NiSO_4$, also inhibited the LC reaction to various extents in the absence of added thiol (Table II). Adding 5 mM DTT to the reaction mixture neutralized the inhibitory effect of Zn^{2+} (Fig. 7).

Neurotoxic or proteolytic activity of the dichain BoNT/A probably requires an initial reduction of the disulfide bond between the LC and the HC (de Paiva *et al.,* 1993). Therefore, the proteolytic assay mixture of

Table II. Effect of Metal Ions and Thiols and Thiol Reagents on the Activity of the rBoNT/A LC

Thiol reagent	Concentration (mM)	% Activity	Metal reagent	Concentration (mM)	% Activity
None[a]		100	EDTA	1	00
Dithiothreitol	5	45	$ZnCl_2$	0.25	60
Dithioerythreitol	5	60		1	10
β-Mercaptoethanol	5	120		0.25	
Glutathione, reduced	5	75		+Dithiothreitol 5	125
Glutathione, oxidized	5	75	$MnCl_2$	1	40
S-Nitrosoglutathione	5	55	$MgCl_2$	1	90
L-Cysteine	5	20	$CaCl_2$	1	75
p-Cl-Mercuribenzoate	0.050	00	$FeCl_3$	1	35
Mercuric chloride	0.013	00	$CoCl_2$	1	90
Iodoacetamide	10	80	$CuSO_4$	1	95
			$NiSO_4$	1	55

[a] The reaction mixture contained only the substrate and the rBoNT/A Lc. Other conditions are as described in Section 2.

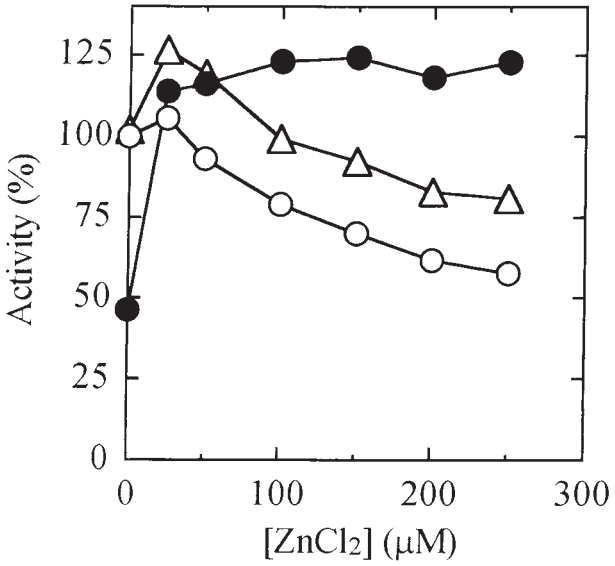

Fig. 7. Inhibition of endopeptidase activity of the rBoNT/A LC by excess Zn^{2+} and protection from inhibition by DTT. The LC was assayed in 50 mM HEPES, pH 7.4, containing 0.9 mM substrate peptide in the absence (-○-) and presence of 5 mM DTT (-●-) or 5 mM mercaptoethanol (-△-) containing the indicated concentrations of $ZnCl_2$. One hundred percent activity (290 nmol/min/mg LC) represents the activity obtained in the absence of any added thiol or Zn^{2+}.

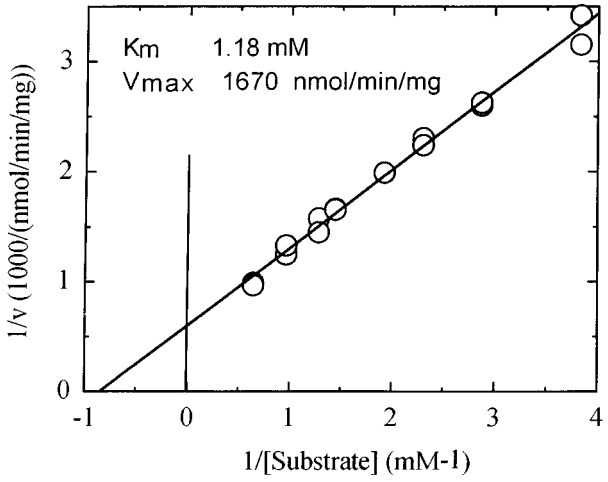

Fig. 8. Determination of K_m and V_{max} from the double-reciprocal (Lineweaver–Burke) plot of initial rates of proteolysis versus substrate concentration by the rBoNT/A LC. The reaction mixtures (0.03 ml) contained 0.25 mM $ZnCl_2$, 0.5 mM DTT, 50 mM HEPES, pH 7.4, and 0.016 mg rBoNT/A LC. The K_m and V_{max} were calculated as 0.9 mM and 1500 nmol/min/mg, respectively.

BoNT/A with the synthetic or natural substrates were supplemented with 5–10 mM DTT (Washbourne *et al.*, 1997; Schmidt and Bostian, 1995, 1997). We found that in the absence of added Zn^{2+}, 5 mM DTT in the reaction mixture significantly inhibited the activity of the LC (Table II and Fig. 7). Similarly, L-cys, dithioerythreitol, and glutathione inhibited the activity to various extents, while β-mercaptoethanol stimulated the activity in the absence of added Zn^{2+}. These results were unexpected as the LC does not posses any disulfide bonds and the invariant Cys responsible for interchain disulfide is far from the active site. One explanation of these effects is the formation of a mixed disulfide between a protein thiol and the exogenous thiol. To investigate the importance of a protein Cys residue on activity, we incubated several sulfhydryl reagents in the proteolytic assay mixture (Table II). Both $HgCl_2$ and *p*-Cl-mercuric benzoate completely abolished the activity of the LC. Preincubating the LC with these two reagents, then diluting with the proteolytic reaction mixture, also gave the same results (data not shown). These results suggest the presence of a protein thiol in the vicinity of the active site of the LC.

3.8. Steady-State Kinetic Parameters

The dependence of reaction rates on the substrate concentration was determined at 0.26–1.7 mM substrate at pH 7.4. A double reciprocal plot of the reaction rates versus substrate concentrations (Fig. 8) yielded a K_m of 1.18 mM and a V_{max} of 1670 (equivalent to 2390 considering a 70% pure LC) nmol/min/mg LC (k_{cat} = 1.39/sec or 1.99 if 70% pure). For comparison, the maximum rate of cleavage of the peptide substrate by the native, dichain toxin is reported to be 1900 nmol/min/mg (k_{cat} = 4.7/sec), while the K_m is 5 mM (Schmidt and Bostian, 1997). The lower K_m for the LC may be due to a more exposed active site in the free LC than in the LC of the dichain, where the active site is shielded from the solvent by elements of the membrane-spanning domain H_n (28–29). The catalytic efficiency k_{cat}/K_m of the rBoNT/A LC, 1.18 (1.69 if 70% pure), is thus higher than that of the dichain, 0.94 (Schmidt and Bostian, 1995, 1997).

3.9. Apo-BoNT/A LC

The rBoNT/A LC was incubated with the metal chelator EDTA and after extensive dialysis, the activity of the apo-BoNT/A LC was measured in the standard reaction mixture. In the absence of any exogenous Zn^{2+} or thiol, the preparation had 15% activity of the holo-BoNT/A LC from which the apoprotein was made (Table III). This result suggests that the bound Zn^{2+} was not completely removed by the EDTA treatment and dialysis. Nonetheless, adding 5 mM DTT and 250 μM $ZnCl_2$ to the assay mixture restored 70% of the activity of the holo-LC. Moreover, in the presence of 5 mM DTT and

Table III. Activities of the Apo-BoNT/A LC with and without Addition of Divalent Metal Ions to the Reaction Mixtures

LC form	Divalent metal	% Activity	% Activity recovered[a]
Holo- LC	$+Zn^{2+}$	100	—
Apo- LC	+None	15	—
	$+Zn^{2+}$	70	65
	$+Mn^{2+}$	20	10
	$+Mg^{2+}$	20	10
	$+Ca^{2+}$	30	20
	$+Fe^{2+}$	0	—

[a] Represents percentage of the lost activity of Zn-free apo-rBoNT/A LC that was recovered by adding the indicated metal ions.

250 μM $MnCl_2$, $MgCl_2$, or $CaCl_2$, 20–30% of the original activity was restored.

4. DISCUSSION

4.1. Overexpression of a Stable, Active, Nontoxic BoNT/A LC

We describe here for the first time a procedure that produces soluble, catalytically and biologically active, stable, gram quantities of the purified LC of a BoNT.

Like a new class of zinc-endopeptidases (Montecucco and Schiavo, 1994), the LC of the BoNTs probably acts by a similar mechanism. Because of their potential role in biological warfare, wide therapeutic applications, and their use as a tool in deciphering the mechanism of membrane exocytosis, the BoNTs and their LCs are targets of vaccine development, drug design, and mechanism studies. In spite of such immense importance, studies of the LC have been limited by its availability. The LC of serotype A has been separated and purified from the full-length toxin by QAE-Sephadex chromatography from 2 M urea; however, the preparation suffers from low solubility (Shone and Tranter, 1995). The LC of serotype C was similarly obtained at a level of <5 mg/10 L culture of *C. botulinum* (Syuto and Kubo, 1981). These preparations almost invariably contain contaminating full-length toxins, and the commercially available preparations precipitate from solution or undergo proteolytic degradation upon hours of storage in solution. More recently the LC of tetanus neurotoxin (Li *et al.*, 1994) and of BoNT/A (Zhou *et al.*, 1995) were expressed in *E. coli* as maltose-binding proteins and purified in 0.5 mg quantities from 1-L cultures (Zhou *et al.*, 1995). However, the poor expression of the cloned products, probably due to rare codon usage in clostridial DNA (Makoff *et al.*, 1989, Winkler and Wood, 1988), remained a major hurdle in obtaining adequate amount of the protein for structural and functional studies. Our strategy was to construct a synthetic gene with *E. coli* codon usage. Growth in a fermentor yielded more than 1g of purified rBoNT/A LC from 1 L of culture. In addition to being soluble, catalytically active, and stable, our preparation of the rBoNT/A LC inhibited neuroexocytosis in sea urchin eggs. Our results of animal vaccination experiments indicate that the rBoNT/A LC is nontoxic to mice and confirms earlier observations that the LC does not have any neutralizing epitope to BoNT/A toxicity (Chen *et al.*, 1997; Dertzbaugh and West, 1996). This latter result contradicts the only report of protective immunity elicited by rBoNT/C LC (Kiyatkin *et al.*, 1997). On SDS–PAGE, the band representing the LC accounted for about 70% of the Coomassie-stained proteins. Partially removing other contaminants under denaturing condition was achieved, but only at the expense of catalytic activity. Because the contaminating proteins were devoid of any proteolytic activity, our preparation provided a convenient source of the LC to study its proteolytic, biological, and structural properties.

4.2. Catalytic Efficiency

BoNT/A cleaves the glutamyl–arginine bond between residues 197 and 198 of the 206-residue SNAP-25. Schmidt and Bostian (1995) showed that a synthetic 17-residue peptide representing residues 187–203 of SNAP-25 was sufficient for detecting endopeptidase activity of BoNT/A and allowing routine assay for the neurotoxin activity. The peptide thus probably mimics the structure of SNAP-25 *in vivo* (Bi *et al.*, 1995). We used the same peptide in an identical method to assay the proteolytic activity of the BoNT/A LC. Substrate K_m for the LC was fourfold lower than that reported for the dichain (Schmidt and Bostian, 1995). This may be due to shielding of the active site by a 'belt' from the translocation domain (H_n) in the dichain neurotoxin (Lacy *et al.*, 1998; Lacy and Stevens, 1999). Thus, the 'belt' may pose a steric hindrance for substrate binding by the dichain (high K_m). Nonetheless, the catalytic efficiency k_{cat}/K_m of the free rBoNT/A LC was somewhat higher than that of the dichain.

An important unanswered question on the mechanism of BoNT action is whether the LC dissociates from the dichain before its proteolytic action. It is often assumed that reducing the disulfide bond between the LC and the HC is a prerequisite for the toxicity of the LC (Schiavo, 1995; Blasi *et al.*, 1993; Schiavo *et al.*, 1993; Rossetto *et al.*, 1994). Stimulating the activity of the rBoNT/A LC, which does not contain a disulfide, by DTT (Fig. 7) suggests additional effects of a reducing

agent. Indeed, a recent report showed a severalfold higher proteolytic activity of a nonreduced BoNT/A-associated protein complex than that of an uncomplexed BoNT/A dichain (Cai *et al.,* 1999). Because the LC by itself can inhibit neuroexocytosis (Bittner *et al.,* 1989) and expression of truncated BoNT/A LC mRNA in *Aplysia* inhibited neurotranmitter release (Kurazono *et al.,* 1992), it is possible that once inside the cytosol, the LC may dissociate from the BoNT/A dichain. The recently determined X-ray structure of the BoNT/A dichain shows a large interaction surface between the LC and the membrane-spanning domain (H_n) besides the 'belt' and the interchain disulfide (Lacy *et al.,* 1998; Lacy and Stevens, 1999). Although evidence for a more flexible conformation of a reduced BoNT/A than the unreduced form has been presented (Cai *et al.,* 1999), the X-ray structure suggests that a large conformational change must accompany a physical dissociation of the LC from the BoNT/A dichain. Our quantitative result of higher catalytic efficiency of the LC appears to indicate that for *in vivo* toxicity, the LC may physically dissociate from the dichain.

4.3. Metal and Thiol Inhibition

Activity of the rBoNT/A LC was partially inhibited by exogenous thiols and was completely inactivated by the thiol-specific reagents $HgCl_2$ and *p*-Cl-mercuribenzoate (Fig. 7, Table I). This suggests a protein thiol in the vicinity of the active site. BoNT/A LC contains three Cys residues (Cys-134, Cys-164, and Cys-430) in the 448-residue sequence. Of these three, only Cys-430 is fully conserved among all BoNT and tetanus neurotoxins and forms the interchain disulfide with another Cys residue in the HC. In the recently determined X-ray structure of the BoNT/A dichain (Lacy *et al.,* 1998; Lacy and Stevens, 1999), Cys-430 and Cys-134 are distant from each other and are located very far from the active site (Fig. 9). Cys-164, on the other hand, is located 9 Å away from the active-site Zn^{2+} and forms part of the wall of the 'pit' leading to the active site. Thiols reversibly form mixed disulfides with a dissimilar thiol and form complexes of varying stability with divalent metal ions, of which the most stable is with mercury (Creighton, 1984). Chemically modifying the thiol of Cys-164 in the LC to form a mixed disulfide (e.g., with DTT) or a stable thiomercurial with a bulkier mercury (e.g., $HgCl_2$) is expected to block the 'pit,' thus restricting substrate access to the active site. By the same token, inhibitory effects of high concentration of Zn^2 probably were neutralized by exogenous thiols or vice versa (Fig. 7, Table II). This may be due to elimination of competition for the protein thiol (Cys-164) by forming an inactive exogenous zinc–thiol complex. Inhibition of BoNT/B LC (Nowakowski *et al.,* 1998) and of other zinc proteases (Auld, 1995) by excess zinc is also known.

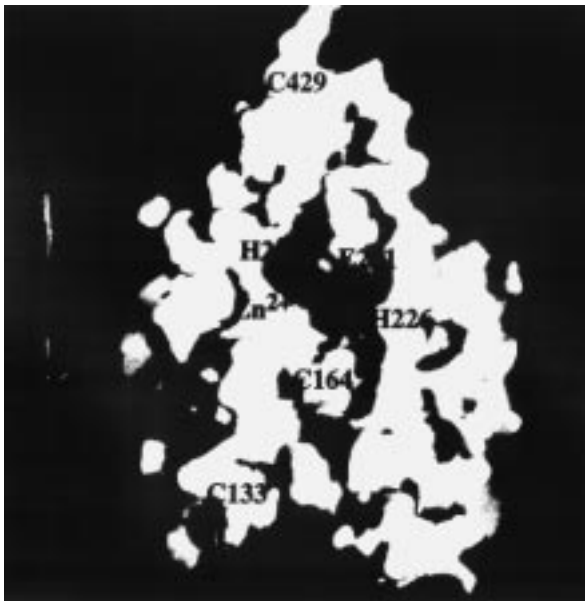

Fig. 9. Location of the three Cys residues in the BoNT/A LC. Molecular surface of the LC portion of the BoNT/A dichain based on its three-dimensional structure (Lacy and Stevens, 1999) is shown. The three Cys residues (yellow), active-site His and asp residues (red), the Zn^{2+} atom (blue) at the active site, and the 'pit' leading to the active site are highlighted. The side chain of Cys-164 lines the surface and forms part of the wall of the 'pit' leading to the active site. The 'pit' acts as an access route of the substrate.

4.4. Flexibility of the Apo-BoNT/A LC

The Zinc-Free rBoNT/A LC retained 15% of the original activity when assayed in the absence of added Zn^{2+}, indicating that zinc was not completely removed, probably due to a tight binding of the zinc to the protein. Tight zinc binding was also inferred from lack of significant stimulation of activity by exogenous zinc in the assay mixture of the holo-LC (Fig. 7). On the other hand, 70% of the activity was restored by adding exogenous zinc to the apo-LC preparation. Activity of the apo-LC was also partially restored by divalent metal ions other than zinc. The rBoNT/A LC thus is similar to many other zinc-peptidases where the catalytic zinc can be replaced by other divalent metals (Auld, 1995). Removing zinc from the LC thus did not cause any deleterious effect on the structure of the protein. Recently Fu *et al.* (1998) reported that reversible removal of zinc from the dichain

BoNT/A produces an irreversible loss of biological activity due to changes in the tertiary structure. Our preparation of the rBoNT/A LC is probably more flexible than a dichain BoNT/A in reversibly accommodating the essential zinc for activity.

In conclusion, we describe a procedure that produces gram quantities of rBoNT/A LC per liter of culture. The protein is highly soluble, nontoxic, catalytically and biologically active, and is stable for a prolonged period, offering the possibility of crystallizing it for structural studies. Availability of this stable preparation allowed us tentatively to identify a Cys residue near the active site that may be a potential target for drug design. Nontoxicity and stability of this preparation should make it an ideal tool to investigate many complex biological processes, e.g., exocytosis and signal transduction.

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