Probing the Mechanistic Role of Glutamate Residue in the Zinc-Binding Motif of Type A Botulinum Neurotoxin Light Chain†

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ABSTRACT: Type A botulinum neurotoxin (BoNT/A) is a zinc endopeptidase that contains the consensus sequence HEXXH (residues 223−227) in the toxic light chain (LC). The X-ray structure of the toxin has predicted that the two histidines of this motif are two of the three zinc-coordinating ligands and that the glutamate is a crucial amino acid involved in catalysis. The functional implication of E224 in the motif of LC was investigated by replacing the residue with glutamine and aspartate using site-directed mutagenesis. Substitution of Glu-224 with Gln (E224Q) resulted in a total loss of the endopeptidase activity, whereas substitution with Asp (E224D) retained about 1.4% of the enzymatic activity (k_{cat} 140 vs 1.9 min^{-1}, respectively). However, K_m values for wild-type and E224D BoNT/A LC were similar, 42 and 50 μM, respectively. Global structure, in terms of secondary structure content and topography of aromatic amino residues, Zn^{2+} content, and substrate binding ability are retained in the enzymatically inactive mutants. Titration of Zn^{2+} to EDTA-treated wild-type and mutant proteins indicated identical enthalpy for Zn^{2+} binding. These results suggest an essential and direct role of the carboxyl group of Glu-224 in the hydrolysis of the substrate. The location of the carboxyl group at a precise position is critical for the enzymatic activity, as replacement of Glu-224 with Asp resulted in almost total loss of the activity.

Botulinum neurotoxins (BoNTs,[1] types A−G) are secreted by *Clostridium botulinum* and are responsible for the neuroparalytic syndrome of botulism characterized by flaccid paralysis. BoNTs are comprised of three subunits: a catalytic light chain (LC), a transmembrane N-terminal half of heavy chain (H_N), and a receptor-binding C-terminal half of heavy chain (H_C). The LC and the heavy chain (HC) are linked through a disulfide bond. While the heavy chain facilitates binding of BoNT to the presynaptic membrane, and translocation of the LC into the cytoplasm, the LC acts as an endopeptidase exclusively on one of the three SNARE (soluble NSF attachment protein receptor) proteins: SNAP-25 (synaptosomal-associated protein of 25 kDa) (*1*, *2*), Syntaxin, or VAMP/synaptobrevin. The cleavage of SNAP-25 prohibits fusion of synaptic vesicles containing acetylcholine, thus blocking the neurotransmitter release.

The endopeptidase activity of BoNTs is unique in many ways, and BoNTs have been described as an entirely new class of Zn^{2+} endopeptidase (*3*). All seven serotypes of BoNT possess an identical Zn^{2+}-binding motif, HEXXH, but are unique either in their substrate selection or in their cleavage site (*2*, *4*). For example, BoNT/A, BoNT/E, and BoNT/C recognize SNAP-25 but cleave at different sites (*1*, *5*, *6*), but BoNT/B recognizes VAMP (vesicle-associated membrane protein) (*2*, *4*, *7*). Another unique feature of BoNT endopeptidases is that their activity is expressed only after their disulfide bond is reduced, except when BoNT is associated with a group of neurotoxin-associated proteins (NAPs) provided by *C. botulinum* (*9*). Furthermore, the bound Zn^{2+} plays both the catalytic and the structural role in the protein (*10*). These and other features of BoNT endopeptidases make them an interesting subject for understanding the mechanistic steps involved in a Zn^{2+} endopeptidase. In addition, an understanding of the molecular features and steps involved in the BoNT cleavage of its substrate is essential for developing antidotes against the neurotoxin.

The crystal structure of type A botulinum neurotoxin (BoNT/A) supports a model in which the H223, H227, and E262 of the HEXXH motif directly coordinate the zinc, and E224 coordinates a water molecule as the fourth ligand (*11*). Based on the analogy to other zinc proteases such as thermolysin, it is believed that Glu-224 in the zinc-binding motif of BoNT/A LC plays a role similar to that of Glu-143 in thermolysin, which is thought to be involved in several steps of peptide bond hydrolysis (*12*). We have investigated

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[1] Abbreviations: BoNT, botulinum neurotoxin; CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; HC, heavy chain; H_C, C-terminal half of heavy chain; H_N, N-terminal half of heavy chain; IAEDANS, 5-((((2-iodoacetyl)amino)ethyl)amino)-naphthalene-1-sulfonic acid; FITC, fluorescein 5-isothiocyanate; ITC, isothermal calorimetry; LC, light chain; NAPs, neurotoxin-associated proteins; SNARE, soluble NSF attachment protein receptor; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel eletrophoresis; SNAP-25, synaptosomal-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein.

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the role Glu-224 by replacing it with Gln, which has a neutral side chain which maintains the side chain length, and with Asp which has the same negative charge but a shortened side chain. These mutants result in either drastically reduced (E224D) or completely abolished (E224Q) endopeptidase activity. The mutations did not introduce any conformational changes in the polypeptide as monitored by circular dichroism and intrinsic fluorescence techniques. The loss of activity was due to interference with the hydrolysis step, and not due to any change in the binding of the SNAP-25 or the Zn^{2+} ligand.

MATERIALS AND METHODS

Oligodeoxynucleotide primers used in constructing mutations and sequencing were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). QuikChange site-directed mutagenesis kit was obtained from Stratagene (La Jolla, CA). Fluorescence probes were obtained from Molecular Probes (Eugene, OR). All other common reagents used were obtained from either Fisher Scientific (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO).

Plasmid Construction and Purification of Recombinant Proteins. The plasmids encoding SNAP-25, BoNT/A LC and LC mutant E224Q, were prepared as described previously (*8, 13*) The SNAP-25 and BoNT/A LC mutants were constructed by site-directed mutagenesis using their respective plasmid as templates, employing the QuikChange kit in accordance with the manufacturer's instructions. For each mutant, a pair of complementary PCR (polymerase chain reaction) primers with 30−40 bases was designed that placed the mutation in the middle of the primers. For SNAP-25 mutant R198T and LC mutant E234D, the forward primers were 5′-GATGAAGCCAACCAAACTGCAACAAAGATG-CTGGG-3′ and 5′-CTCTGGCACACGACCTCATCCACGC-3′, respectively. PCR was performed under the following conditions: initial denaturation for 30 s at 95 °C, 12 cycles of 30 s at 95 °C, 1 min at 55 °C, and 12 min at 68 °C**.** After digestion of the parental DNA with *Dpn*I, the amplified plasmids were transformed into *E. coli* XL-1 B supercompetent cells (Stratagene, La Jolla, CA). The presence of the intended mutation was confirmed by DNA sequencing.

Recombinant LC and LC mutants were produced and purified as described earlier (*13*). Expression of SNAP-25 and its mutants was induced for 3 h of incubation at 37 °C, and proteins were isolated on a His-Bind (Novagen, Inc., Madison, WI) column according to the manufacturer's instructions.

Circular Dichroism Spectroscopy. CD data were collected with a JASCO J-715 spectropolarimeter equipped with a computer-controlled temperature cuvette holder. Far-UV CD spectra in the region 180−250 nm were recorded with a 1.0 mm path length cell containing 0.1−0.3 mg/mL protein in 20 mM Tris buffer, pH 7.0, containing 50 mM NaCl. Near-UV CD spectra (240−310 nm), representing the average of four scans, were obtained with a 1 cm cell containing 0.5−1.0 mg/mL protein in the same buffer. All spectra were corrected for the buffer. Both far-UV and near-UV CD spectra were recorded at room temperature (25 °C) at a speed of 20 nm/min with a response time of 8 s.

The thermal denaturation studies were performed by monitoring the CD signal at 222 nm. The temperature was increased with a heat rate of 2 °C/min from 30 to 80 °C.

Fluorescence Measurement. Tryptophan fluorescence lifetime was measured using an ISS K2 fluorometer (ISS, Inc., Champaign, IL). The excitation wavelength was 295 nm, and protein samples with a concentration of 0.1−0.2 mg/mL were used. Excitation and emission slits were 8 nm, and a 335 nm cutoff filter was used in the emission pathway. Phase and modulation values were determined relative to a Terphenyl (in ethanol) reference solution, which has a lifetime of 1.05 ns. Twelve frequencies were used between 2 and 200 MHz in the log mode, with a minimum of 5 and a maximum of 10 measurements at each frequency before averaging the signal. The temperature of the sample was kept at 10 °C by a circulating bath.

Zinc Content of Wild-Type and Mutant LC. For zinc analysis, the protein samples were extensively dialyzed against 20 mM Tris buffer (pH 7.0 or pH 4.7), containing 50 mM NaCl in metal-free dialysis tubing prepared according to Fu et al. (*10*). The concentration of residual zinc in the buffer was subtracted from those in the targeted protein samples. Graphite furnace atomic absorption spectrometry (Perkin-Elmer 5100 PC, Norwalk, CT) was used for zinc analysis. Absorbance peaks were measured at 213.9 nm. All values shown are averages of duplicate determinations.

Endopeptidase Assay. The SNAP-25 cleavage experiments were carried out to analyze the enzymatic activity of the LC and its mutants. The cleavage was determined by monitoring the decrease in band intensity of SNAP-25 on a 10% sodium dodecyl sulfate−polyacrylamide gel eletrophoresis (SDS−PAGE) gel by incubating a series of SNAP-25 concentrations (40, 27, 18, and 12 μM) with 20 nM LC or 2.2 μM E224D-LC in a 10 μL reaction mixture for 5 min at 37 °C. The reaction buffer was 20 mM Tris, pH 7.0, containing 50 mM NaCl and 1 mM DTT. The cleavage was terminated by addition of SDS−PAGE sample buffer to the mixture. For resolving the cleavage products, the discontinuous electrophoresis was carried out with the Mini Protein II (BioRad, Hercules, CA) at a constant voltage of 110 V. The gel was stained with Coomassie blue, and scanned for integrating the band density using an *itti* Imager (itti, I.c., St. Petersburg, FL). To determine the initial rate of SNAP-25 cleavage, the SNAP-25 band was integrated for density in respective lanes with different concentrations of SNAP-25 used in the reaction mixture, and compared with the integrated density of the control SNAP-25 (without LC).

Binding between BoNT/A Light Chain and SNAP-25. Binding of wild-type and mutant LC with SNAP-25 was analyzed by monitoring energy transfer between fluorescent probes attached to the LC and SNAP-25. Wild-type and mutant LC were labeled at Cys with 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS); wild-type and mutant SNAP-25 were labeled at Lys with fluorescein 5-isothiocyanate (FITC) in the dark following the procedures suggested by Molecular Probes (Eugene, OR). Briefly, the reduced (with 10 mM dithiothreitol) wild-type and mutant LC (1 mg/mL) were dialyzed overnight against 10 mM phosphate buffer containing 50 mM NaCl, pH 7.3, to remove DTT (dithiothreitol), followed by reaction with a 10-fold molar excess of IAEDEANS for 2 h at 25 °C in the dark. Stock IAEDANS (5 mg/mL) was prepared in DMF (dimethylformamide). The reaction was terminated by addition of 10 mM DTT. SNAP-25 and its mutant were labeled by incubating them with a 20-fold molar

excess of FITC (5 mg/mL stock in DMF) in 0.05 M borate buffer, pH 9.0, containing 50 mM NaCl. After 2 h, the reaction was quenched by the addition of freshly prepared 1.5 M hydroxylamine (pH 8.5) to a final concentration of 0.15 M. Both reaction mixtures were dialyzed separately against 20 mM Tris, pH 7.0, containing 50 mM NaCl in order to remove free dyes.

The labeling ratio for IAEDANS was determined as follows. The attached dye concentration was determined from the absorbance at 337 nm by using ϵ_{337}(IAEDANS) = 6.1 mM^{-1} cm^{-1} (*15*). The absorbance due to the label at 280 nm was then calculated by using ϵ_{280}(IAEDANS) = 1.06 mM^{-1} cm^{-1}. The absorbance due to LC or its mutant was obtained from the total absorbance minus the absorbance due to the label at 280 nm. The concentration of the LC was calculated by using a specific absorbance of ϵ_{280}(LC) = 0.83 mg^{-1} cm^{-1} and molecular mass of 52.67 kDa. Most of the experiments were performed on LC or its mutant with a labeling ratio of 1.2 ± 0.2 mol of IAEDANS per mole of protein. The calculation of the labeling ratio for FITC was similar to the IAEDANS, with absorption coefficients of ϵ_{493}(FITC) = 74.5 mM^{-1} cm^{-1} (*16*), ϵ_{280}(FITC) = 29.8 mM^{-1} cm^{-1}, ϵ_{280}(SNAP-25) = 0.80 mg^{-1} cm^{-1}, and a molecular mass of 24.72 kDa for SNAP-25.

The donor fluorescence quenching was measured by titrating FITC-SNAP-25 with IAEDANS-E224Q LC at a fixed temperature of 10 °C. The sample was gently mixed by pipeting up and down 5 times, and allowed 1 min for the binding. Excitation was fixed at 360 nm, and fluorescence emission spectra were recorded between 400 and 600 nm. To determine the dissociation constant, K_d, a fixed concentration of IAEDANS-E224Q LC (0.59 μM) was titrated with different concentrations of FITC-SNAP-25 (0.075−1.35 μM), and the fluorescence intensity of IAEDANS-E224Q LC was measured at 460 nm where no interference occurs from the fluorescence of FITC.

The results from the spectrofluorometric titration were analyzed by Scatchard plot (*16*) to evaluate the binding parameters for SNAP-25 binding with BoNT/A LC. The concentration of bound SNAP-25, which is equal to the concentration of bound E224Q-LC ($[E224Q\text{-}LC]_b$), was obtained at each substrate concentration from the ratio:

$$[E224Q\text{-}LC]_b/[E224Q\text{-}LC]_o = \Delta F/\Delta F_{max}$$

on the assumption that the fluorescence change is directly proportional to the amount of binding. $[E224Q\text{-}LC]_o$ is the concentration of total E224Q-LC in the reaction mixture. ΔF is the change in fluorescence emission intensity at 460 nm (λ_{ex} = 360 nm) upon addition of each aliquot of FITC-SNAP-25, and ΔF_{max} is the same parameter when all the E224Q-LC molecules were bound to SNAP-25 (saturating condition).

Titration Calorimetric Analysis of Zn^{2+} Binding to the Light Chain. Apo-protein of light chain was prepared as described previously (*10*), except the binding buffer used in this study was 20 mM Tris, 50 mM NaCl, pH 7.0. The binding isotherms of Zn^{2+} to the apo-LC were generated employing CSC Isothermal Titration Calorimetry (Calorimetry Science Co., Provo, UT) at 25 °C. After temperature equilibration, the binding was measured by titrating 5 μL injections of 5 mM ligand $ZnSO_4$ in the reaction buffer (20 mM Tris, 50 mM NaCl, pH 7.0) into the protein solution.

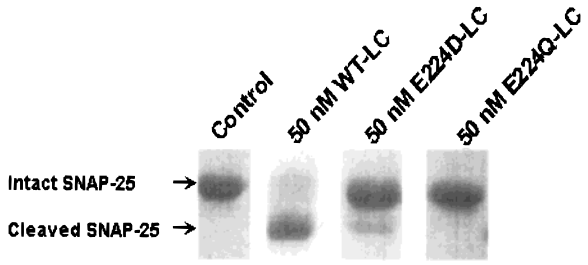

FIGURE 1: Comparison of endopeptidase activity of wild-type LC and its mutants. 20 μM SNAP-25 was incubated with 50 nM WT-LC or mutant for 1 h at 37 °C. Control SNAP-25 sample as well as its products were analyzed on a 10% SDS−PAGE gel. Enzymatic activity was dramatically reduced in E224D-LC and completely abolished in E224D-LC.

Protein samples ranging in concentration from 20 to 30 μM in a total volume of 1.3 mL were placed in the reaction cell. The observed heat change accompanying titration was measured after each injection. The total observed heat effects were corrected for the heat of dilution of ligand by performing control titrations in the buffer used for dissolving the protein. The resulting titration curves were deconvoluted for the best-fit model using Titration BindWorks in the ITC software package (CSC, Provo, UT) to obtain the affinity constant and the number of binding sites.

RESULTS

Enzymatic Activity of WT-LC, E224Q-LC, and E224D-LC. Replacement of Glu with Gln totally abolished the endopeptidase activity of LC against SNAP-25, as shown in Figure 1. An increase in the concentration of the mutant LC or prolonged incubation did not exhibit any further cleavage of SNAP-25 (data not shown). Mutation of Glu to Asp dramatically impaired but did not abolish the endopeptidase activity as evidenced by the much less intensive band corresponding to cleaved SNAP-25 (Figure 1). At 50 nM concentration, wild-type LC cleaved about 95% of SNAP-25 in 1 h whereas E224D LC cleaved only about 5% of SNAP-25.

Enzyme Kinetics. The enzyme kinetic analysis was carried out by estimating the cleavage of SNAP-25 by wild-type and mutant proteins for 5 min, and then calculating the initial velocity of the reaction on a per minute basis. A Lineweaver−Burk plot was constructed (Figure 2), and K_m and V_{max} were derived from the intercept and slopes, respectively. The enzyme kinetic parameters of wild-type LC and its mutants are summarized in Table 1. WT-LC and the E224D mutant protein displayed k_{cat} values of 140 and 1.9 min^{-1}, respectively, while E224Q displayed no endopeptidase activity. In contrast, the K_m values for the mutant E224D-LC were similar to the wild-type LC. Thus, while alterations in the side chain of Glu-224 dramatically affected LC's endopeptidase activity, it did not seem to interfere with the productive substrate binding.

It has been reported that BoNT/B light chain cleaves VAMP peptide 60-94 with a K_m of 346 μM and a k_{cat} of 26.3 s^{-1} (or 1578 min^{-1}) (*17*), while tetanus toxin light chain cleaved VAMP 1-93 with a K_m of 192 μM and a k_{cat} of 0.5 min^{-1} (*18*). A relatively smaller value of K_m (41 μM) was observed in SNAP-25 cleavage by BoNT/A light chain (Table 1). The K_m for a 17-residue SNAP-25 peptide

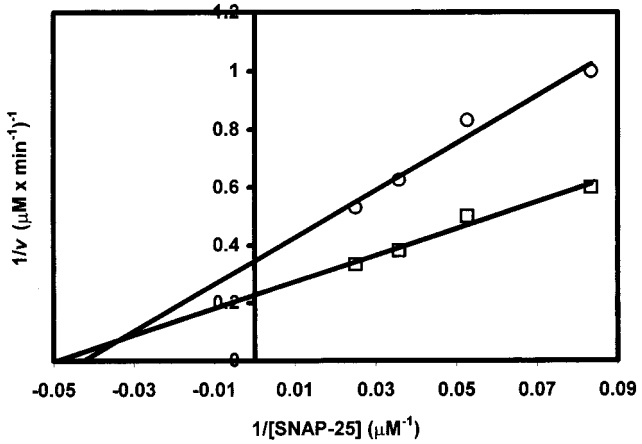

FIGURE 2: Lineweaver−Burk plots of cleavage of SNAP-25 by WT-LC (20 nM) (circles) and by E224D-LC (2.2 μM) (boxes) for 5 min at 37 °C. A series of SNAP-25 concentration (40, 27, 18 and 12 μM) were used.

Table 1: Enzyme Kinetic Parameters for Wild-Type LC (WT-LC) and Its Mutants (E224-LC, E224Q-LC)

enzyme	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
WT-LC	41 ± 10	140 ± 51	3.2
E224D-LC	50 ± 12	1.9 ± 0.6	0.04
E224Q-LC		nd[a]	nd

[a]nd: not detectable.

cleavage by BoNT/A was estimated as 1.7 mM (*19*). The lower K_m value observed for SNAP-25 cleavage might be related to the requirement of full length of the substrate for optimal binding. The difference in k_{cat} values perhaps reflects on the differences among different toxins and substrates used, vis-à-vis their activity with different substrates. Ours is the first report of enzyme kinetic parameters with intact substrate of BoNT endopeptidase activity. However, due to the nature of the electrophoretic analysis of cleavage product, our initial velocity estimates are based on a 5 min cleavage data. Such estimates may not reflect true initial velocities of the reaction.

Structure Analysis. CD spectra were recorded in the far-UV and near-UV regions so that secondary structure elements of the protein and the conformational environment of the aromatic residues could be illustrated. To examine whether the mutation Glu-224 to Gln or Asp had disrupted the enzyme's secondary structure, far-UV CD spectroscopy was employed. Wild-type and mutant LC showed a CD spectrum typical of α-helical protein with double minima at 208 and 222 nm (Figure 3, top). The near-UV CD spectrum displays a broad minimum in the tyrosine region at 280 nm and two distinct minima at 263 and 269 nm in the phenylalanine region (Figure 3, bottom). The spectra of the two mutants, E234Q and E234D, are virtually indistinguishable from that of the wild-type protein in both the near- and the far-UV region (Figure 3), thus excluding alteration of LC's overall structural folding by the mutation.

In a further effort to probe any conformational alteration introduced by the replacement of Glu-224 with Gln or Asp, we examined Trp fluorescence characteristics. Frequency response curves of wild-type LC and the mutants (Figure 4) recorded for tryptophan lifetime measurements were essentially superimposable, implying that the replacement of the Glu-224 with Gln or Asp did not evoke any topographical

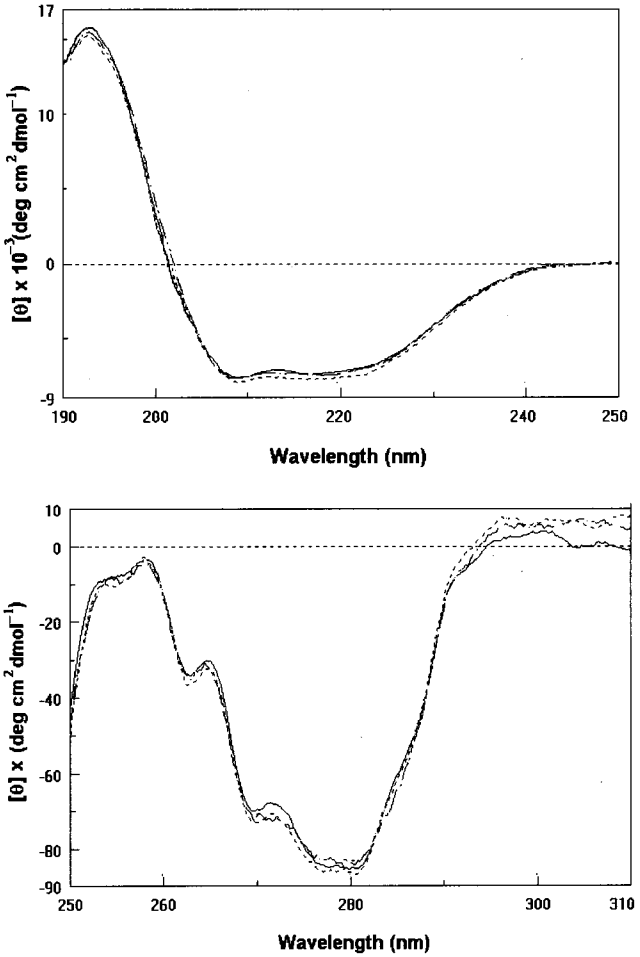

FIGURE 3: Circular dichriosm spectra of WT-LC (solid curves), E224Q-LC (dashed curves), and E224D-LC (dotted curves) in the far- (top) and near-UV (bottom) regions. 0.1−0.3 mg/mL protein samples in 20 mM Tris buffer (pH 7.0), containing 50 mM NaCl, were used for far-UV CD measurement. 0.5−1.0 mg/mL protein samples in the same buffer were used for near-UV CD measurement. The spectral contributions of buffer were subtracted from the protein spectra.

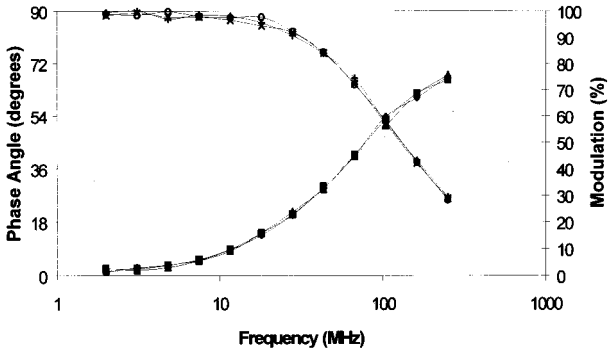

FIGURE 4: Frequency response curves of WT-LC [(■) phase, (+) modulation], E224Q-LC [(◆) phase, (×) modulation], and E224D-LC [(▲) phase, (○) modulation] with excitation at 295 nm. A 335 nm cutoff filter was used in the emission pathway.

or microenvironmental changes around the two Trp residues in the LC. This observation again confirmed our conclusion above that the mutation did not induce any conformational change.

The temperature-induced denaturation curves of LC and the two mutants are virtually identical (Figure 5), which is consistent with a same overall protein folding in all three

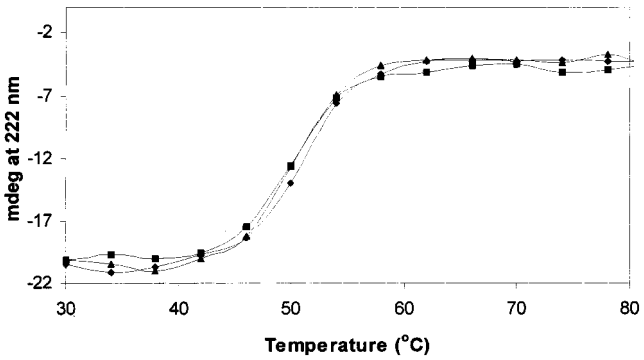

FIGURE 5: Temperature denaturation of WT-LC (■), E224Q-LC (◆), and E224D-LC (▲) as monitored by the CD signal at 222 nm. The temperature was raised at a rate of 2 °C/min. The concentration of W-LC, E224Q-LC, and E224D-LC was 0.29 mg/mL.

proteins. The data confirm that no dramatic conformational changes occurred as a result of mutation of E224 to D224 or Q224.

Substrate Binding Analysis by Fluorescence Resonance Energy Transfer. LC and its mutant E224Q were labeled with IAEDANS at a molar ratio of 1.2:1 (probe:protein) at 1 of the 4 cysteine residues, while SNAP-25 and its mutant R198T were labeled with FITC at 2 out of 35 Lys residues in each protein molecule. SNAP-25 mutant R198T (R198T being the P_1' position of the cleavage site) was employed here since it was not readily cleaved by the LC. In contrast, SNAP-25 mutant Q197L was readily cleaved by the LC (data not shown). The E224Q mutant LC was used in the binding to the wild-type SNAP-25 to avoid any cleavage reaction during the measurement. To obtain substrate binding affinity, donor quenching was measured by titrating FITC-SNAP25 with IADEANS-E224Q. The decrease in donor fluorescence and increase in acceptor emission indicated energy transfer between the two probes (Figure 6). The fluorescence intensity of IADEANS-E224Q was measured at 469 nm where no contribution of the fluorescence from FITC occurred. The parameters of substrate binding (n and K_d) were calculated from the double-reciprocal plot (Figure 6; Table 2). The results suggested similar binding affinity (K_d, 0.10−0.12 μM) regardless of alternation of the active Glu-224 in the enzyme or scissile bond in the substrate. The number of binding site was estimated as 1, suggesting a homogeneous binding between the two proteins.

Characterization of Zn^{2+} Binding Affinity. Although we found similar zinc content in wild-type LC and its mutant E224Q (Table 3), the possibility still existed that the diminished activity of E224Q could result from a change in the Zn^{2+} binding affinity to the active site. To assess this possibility, we employed isothermal titration calorimetry (ITC) to monitor the heat changes that accompany Zn^{2+} binding to apo-WT/LC and apo-E224Q/LC.

We first prepared EDTA-free apo-protein by extensive dialysis of the EDTA-treated protein sample for 48 h. We observed that the apo-LC seemed to aggregate more readily than holo-LC, since precipitation occurred during the removal of EDTA. We therefore changed the dialysis time to 24 h, and used dialysis tubing with larger pore size and employed frequent changes of the dialysis buffer. For each set of experiments with both apo-LC and apo-E224Q/LC, both

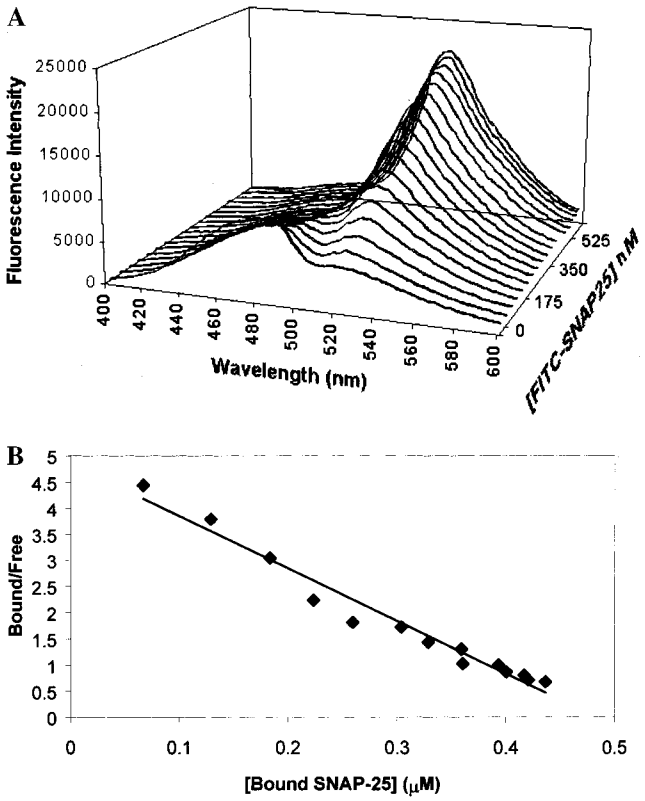

FIGURE 6: Fluorescence titration of AEDANS-labeled E224Q/LC with FITC-labeled SNAP-25. (A) Sequential addition (5 μL) of 15 μM FITC-SNAP-25 stock solution to a cuvette containing 1 mL of 0.59 μM AEDANS-E224QLC. Traces correspond to the emission spectrum of a mixture of AEDANS-E224QLC with a series of concentrations of FITC-SNAP-25, with the excitation wavelength fixed at 360 nm. (B) Scatchard plot for the binding of FITC-SNAP-25 to AEDANS-E224Q/LC.

Table 2: Substrate Binding of Wild-Type LC and Its Mutant

	K_d (μM)	n (mol/mol)
SNAP-25 to LC/E224Q	0.10	0.8
SNAP-25/R198T to LC	0.12	0.8
SNAP-25/R198T to LC/E224Q	0.11	0.9

Table 3: Zinc Content of BoNT/A LC, Wild Type, and Mutants[a]

protein	Zn^{2+} (mol/mol of protein)
wild type	1.11 ± 0.21
E198D	1.22 ± 0.26

[a] Standards containing zinc ranged from 15.4 to 92.4 nM. Measured values ranged from 18.5 to 65.2 nM.

samples were prepared and titrated on the same day under the same instrumental setup conditions. Final data were the average of three sets of experiments.

In the concentration range used for the experiments, 20−30 μM apo-LC samples, the stoichiometry for the Zn^{2+}-LC process is ~0.8:1 (Figure 7; Table 4). At 25 °C, the enthalpy change for Zn^{2+} binding was large and endothermic, $\Delta H = 23 \pm 3$ kcal mol^{-1}, and the affinity constant k was calculated to be 5.1×10^4 M^{-1}. Binding of Zn^{2+} to the mutant E224Q showed a similar affinity with $K = 4.6 \times 10^4$ and binding enthalpy of $\Delta H = 21 \pm 4$ kcal mol^{-1}, indicating the contribution of Glu-224 to the binding strength of Zn^{2+} in the active site is negligible compared to other coordinating residues.

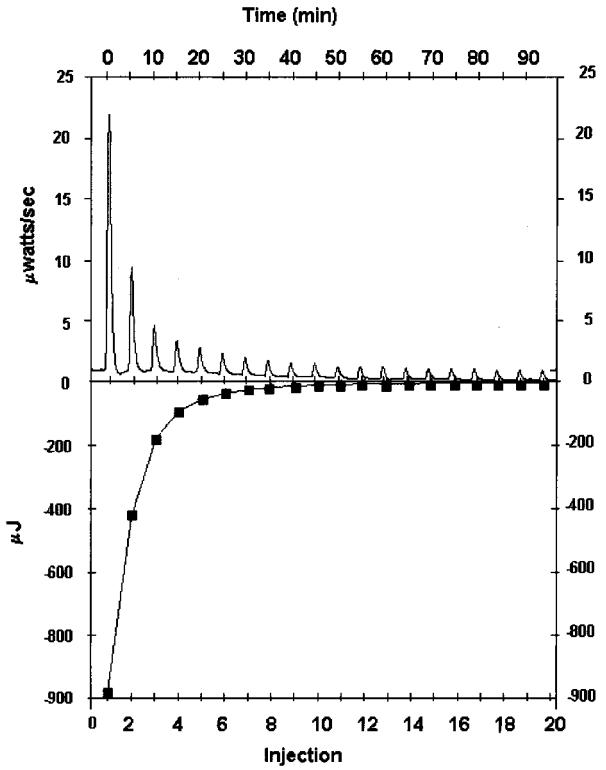

FIGURE 7: Calorimetric titration of apo-LC with $ZnSO_4$. The ligand (5 μL injections) was added to the protein solutions at 300 s intervals. The titrations were performed at 25 °C in a metal-free buffer containing 20 mM Tris, 50 mM NaCl, pH 7.0. The upper panel shows the power−time curve from the titration experiment. The lower panel is the plot of processed microcalorimetric data where correction for enthalpy of dilution of $ZnSO_4$ has been made. The solid line corresponds to the best-fit curve obtained by a nonlinear regression.

Table 4: Binding Parameters of Zn^{2+} to Apo-WT/LC and Apo-E224Q/LC As Determined by ITC

ΔH (kcal mol^{-1})	ΔH (kcal mol^{-1})	K (M^{-1})	n (mol/mol)
apo-WT/LC	23 ± 3	5.1×10^4	0.8
apo-E224Q/LC	21 ± 4	4.6×10^4	0.85

DISCUSSION

Botulinum neurotoxin is an enzyme of enormous interest, because of its unique features of substrate specificity, cleavage site selectivity, and mode of activation, and because of its relevance to food poisoning disease botulism, biological weapons, and therapeutics against neuromuscular disorders (*20*). The crystal structure of BoNT/A has recently been published (*11*), and active site residues have been identified. This communication focuses on the conformational characterization of the BoNT/A LC, which is the toxic portion of the neurotoxin, and on the examination of the functional role of Glu-224 in the endopeptidase activity of BoNT/A LC.

We have altered the key amino acid residue Glu-224 of the Zn^{2+}-binding motif, HEXXH, in the active site of LC to two different residues: Gln, a noncharged residue with addition of an amino group; and Asp, which maintains a negative charge at the same position. The altered enzymes were compared to wild-type LC with respect to zinc content, substrate binding, and enzyme kinetic parameters (in the case of E224D). These mutants were not devoid of Zn^{2+} as shown by atomic absorption analyses (Table 3). The K_m value of the substrate for E224D was almost identical to the K_m for the wild-type LC, indicating that mutating E224 does not result in any major change in substrate binding. Despite identical binding to the substrate, E224 mutants lost their catalytic activities. E224Q-LC lacked any endopeptidase activity, whereas E224D retained only about 1.4% enzymatic activity compared to the wild-type LC (Table 1). The catalytic efficiency, k_{cat}/K_m, was dramatically reduced from 3.2 to 0.04 μM^{-1} min^{-1}, an 80-fold decrease. The fact that mutant LC retains its structural folding and its binding affinity to SNAP-25 but still loses its enzymatic activity indicates that E224 directly participates in the catalytic process. It seems that for endopeptidase activity, a strict topographical requirement of active site residues is maintained. This is reflected in a dramatic reduction of the enzymatic activity of E224D, which is a conservative change with only 1.4 Å difference in the side chain length. This observation is consistent with our earlier results which suggested that the perturbation of active site folding as a result of Zn^{2+} removal is not easily restored (*10*). The question remains whether Zn^{2+} binding in terms of its affinity remains unaffected by the BoNT/A LC.

To gain a comprehensive quantitative assessment of Zn^{2+} binding, we have employed isothermal titration calorimetry for monitoring the heat changes that accompany metal ion binding to the wild-type and mutant LC. In addition to K_d and stoichiometry, values for ΔH and the number of binding sites, n, can also be extracted from an ITC profile. We observed virtually no difference in the derived enthalpy changes and binding constants in the wild type and E224Q, indicating that there is no detectable change in zinc binding affinity to the active site. The results presented herein for Zn^{2+} binding, when combined with data derived from structure and activity study, illustrate the loss of activity provoked by these mutations was not due to major perturbations of the active site, but resulted from the replacement of the negatively charged Glu.

BoNT/A LC is thought to have an active site organization that closely resembles that of thermolysin (*21*), another zinc endopeptidase. E224 is in a position to be protonated during the course of the reaction via the abstraction of a proton from an attacking water, a function attributed to E143 in thermolysin (*23*−*25*). The pK_a of the side chain carboxylate of glutamate is 4.2. At the pH of enzyme activity, 7.0, this residue would be negatively charged, unless its pK_a has been altered by the environment of the active site. Removal of the carboxylate residue at this position in LC caused a complete loss of catalytic activity in E224Q but no significant change in the dissociation constant K_d for SNAP-25. Thus, the putative hydrogen bond of the potentially dissociable proton of water to the lone electron pair of Glu-224 is probably not important in substrate binding but is important for catalysis as indicated by the diminished endopeptidase activity (Figure 1). These experiments confirm that a glutamate residue plays a mechanistic role in the hydrolysis step. Our data, in conjunction with the crystal structure published recently (*11*), suggest a putative reaction mechanism (Figure 8) by which BoNT/A LC cleaves SNAP-25: a water molecule is displaced from the zinc atom by the carbonyl oxygen of the substrate and then polarized by the carbonyl carbon of the scissile peptide bond. Simultaneously, a proton is transferred to the nitrogen of the peptide bond

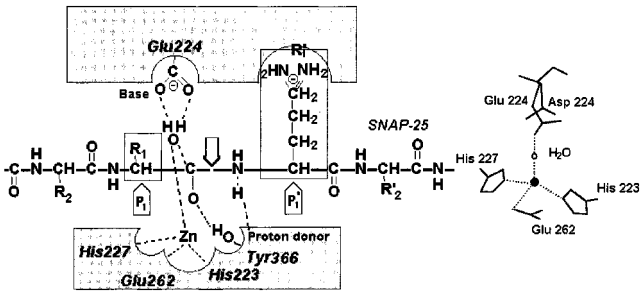

FIGURE 8: Left: Putative reaction mechanism for the hydrolysis of SNAP-25 by BoNT/A LC. The zinc atom is coordinated to three zinc ligands (His-223, His-227, and Glu-262) and a water molecule. The negative charge of Glu-224 is the nucleophile and shuttles the proton to the leaving group. Right: zinc binding site of wild-type LC and mutant E224D.

from a proton donor in the LC. Tyr-366, which might be equivalent to Tyr-157 in thermolysin (*25*, *26*), is likely to be involved in substrate binding, based on its close location to the zinc atom (*11*).

Asp retracts the carboxyl group by a distance of approximately 1.4 Å due to shortening of the side chain by a $-CH_2-$ group. Several hypotheses can be proposed to account for the reduction but not elimination of the endopeptidase activity of E224D-LC. The formation of a hydrogen bond between the substrate and other active site residues along with Glu-224-bound water might be required to limit the rotation of the cleaving segment, while Asp-224 might not be able to allow optimal interaction between the oxygen atom of the water and the scissile bond. Alternatively, the possible loss of a structured water molecule present in the E224D-LC could be unfavorable for the hydrolysis reaction.

In summary, we have demonstrated that (i) even a conservative substitution of Glu-224 in the HEXXH motif of BoNT/A endopeptidase domain results in the loss of enzymatic activity, (ii) loss of the enzymatic activity in E224Q or E224D mutants of BoNT/A LC is not due to any alteration in the polypeptide folding, (iii) the carboxyl groups of Glu-224 directly participate in the hydrolysis of the substrate peptide without affecting the Zn^{2+} binding to the active site, and (iv) the topographical positioning of the carboxyl group is extremely critical for the peptide hydrolysis.

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