The Complete Amino Acid Sequence of the *Clostridium botulinum* Type D Neurotoxin, Deduced by Nucleotide Sequence Analysis of the Encoding Phage d-16ϕ Genome

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ABSTRACT. The complete nucleotide sequence of *Clostridium botulinum* type D strain CB16 neurotoxin was determined and the deduced amino acid sequence is reported here for the first time. The structure and function of botulinum type D neurotoxin is discussed from a molecular biological viewpoint. DNA was extracted from toxin-converting phage d-16ϕ of *C. botulinum* type D strain CB16, and a fragment (about 10 kbp) coding for the neurotoxin was cloned into *Escherichia coli* using λgt11. A 21-mer oligonucleotide which corresponds to Phe^{7} to Val^{13} of the partial amino acid sequence near the N-terminus of the type D neurotoxin was synthesized and used as a probe to identify the gene encoding type D neurotoxin. The nucleotide sequence contained a single open reading frame coding for 1,275 amino acids (molecular weight of 146,785) and the deduced amino acid sequence corresponded exactly to the partial amino acid sequences determined by direct microsequencing of the neurotoxin fragments. In the dichain molecule of the neurotoxin, Thr^{2} and Asn^{443} formed the N-termini of the light chain (M.W. 50,410) and heavy chain (M.W. 96,394) respectively, and these two chains were linked with a disulfide bond between Cys^{437} on the light chain and Cys^{450} on the heavy chain. The nucleotide sequence of the D-CB16 neurotoxin differed from that previously reported for type D neurotoxin by three nucleotides.—KEY WORDS: amino acid sequence, botulinum neurotoxin, *Clostridium botulinum*, neurotoxin, nucleotide sequence.

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Clostridium botulinum has seven toxin types, A through G, differentiated by the antigenic specificity of the neurotoxins. Of these seven types, only type C and D have been determined their toxigenicity by specific bacteriophages [5, 10]. These phages, named "toxin-converting phages" are heterogeneous in their host specificity and antigenicity, and are divided into four groups according differences in these characteristics [17, 23]. The neurotoxins produced by botulinum type C and D strains are also antigenically complex [19]. These neurotoxins are considered to consist of antigenic sites common to both type C and D toxins, as well as type specific antigenic sites [18–20]. Furthermore, type C and D toxins have been found to consist of a mosaic of strain-specific epitopes and epitopes common to the toxin types, respectively [16, 20]. The heterogeneity of type C and D neurotoxins is presumed to result from the mutation and recombination of phage genomes controlling type C and D neurotoxin production [18, 26]. To illustrate the heterogeneity of these type C and D neurotoxins, knowledge of the complete structure of these neurotoxins is necessary. Recently, the complete nucleotide sequences of the structural genes of botulinum type C and D neurotoxins have been reported [1, 8, 11].

A type D converting phage d-16ϕ was isolated then biologically and biophysically characterized from a toxigenic strain D-CB16 isolated in Japan. Results indicated that the phage was different from those previously isolated on the basis of host specificity and antigenicity [23, 24]. In this paper, the botulinum type D neurotoxin gene from phage d-16ϕ DNA was cloned and the complete nucleotide sequence of botulinum type D neurotoxin was determined. Furthermore, the amino acid sequence of type D neurotoxin deduced from the nucleotide sequence was confirmed to include identical amino acid sequences to those determined by direct microsequencing of light and heavy chains of type D neurotoxin and polypeptides obtained from the neurotoxin after limited proteolysis with endopeptidase. The deduced amino acid sequence of botulinum type D neurotoxin is reported here for the first time, and compared with previously reported amino acid sequences of botulinum type A, C and tetanus neurotoxins [2, 4, 7, 11, 27].

MATERIALS AND METHODS

Bacterial strains and phage: Clostridium botulinum type D toxigenic strain CB-16 was provided by

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T. Itoh, Tokyo Metropolitan Research Laboratory of Public Health, and was used for the preparation of type D neurotoxin. The toxin converting phage d-16ϕ was derived from strain CB-16 by UV irradiation [23] and was cloned by three successive single plaque isolations with the indicator strain D-16nt.

Purification of type D neurotoxin: Botulinum type D neurotoxin from type D toxigenic strain CB-16, was produced by the dialyzing cultivation method reported by Syuto and Kubo [25] with some modifications. The toxic protein in the culture supernatant (500 m*l*) was precipitated with ammonium sulfate at a 50% saturation. The precipitate was dissolved in 50 m*l* of 1 M NaCl-50 mM acetate (pH 4.0) buffer, and dialyzed against one *l* of 0.2 M NaCl-50 mM acetate (pH 4.0) buffer for three times. The crude toxin was adsorbed to a SP-Toyopearl (Tosoh, Tokyo, Japan) column (1.5 × 40 cm) equilibrated with dialyzing buffer. After the column was washed with the same buffer, total volume of 1,000 m*l* of linear 0.2–0.8 M NaCl gradient in 50 mM acetate (pH 4.0) buffer was used to elute the toxin. The toxic fractions were bufferized at pH 7.8 with 20 mM Tris-HCl (pH 7.8) buffer by ultrafiltration using an Amicon YM30 membrane (Amicon, Danvers, MA, U.S.A.) and concentrated to minimum volume (about 5 m*l*). The neurotoxin was adsorbed to Mono Q HR 5/5 column equilibrated with 20 mM Tris-HCl (pH 7.8) buffer attached to FPLC (Pharmacia LKB, Uppsala, Sweden) and eluted from the column with a stepwise gradient of 20 mM Tris-HCl (pH 7.8) buffer to 0.4 M NaCl-20 mM Tris-HCl (pH 7.8) buffer.

Preparation of peptides from purified neurotoxin by limited proteolysis with endopeptidase: The native neurotoxin was heated for 5 min at 95°C in the presence of 0.5% sodium dodecylsulfate (SDS), 0.5% mercaptoethanol and 10% glycerol, and subjected to SDS-PAGE with 7.5% gel according to Laemmli [13]. After staining with Coomassie Blue, the bands for the light and heavy chains of the neurotoxin were cut out from SDS-polyacrylamide gel and then digested with *Staphylococcus aureus* V8 protease (Wako Pure Chemical, Osaka, Japan) or *Achromobacter lyticus* lysyl endopeptidase (Wako Pure Chemical, Osaka, Japan) by placing gel slices in the wells of the second SDS-PAGE. Digestion proceeded directly in the stacking gel during the subsequent electrophoresis according to Cleveland *et al.* [3].

N-Terminal amino acid sequence of the neurotoxin fragments and peptides: The separated light and heavy chains of the neurotoxin, and their derivative peptides were electrotransferred to a ProBlott™ polyvinylidine difluorid membrane (PVDF; Applied Biosystems, Foster City, CA, U.S.A.) in transfer buffer [25 mM Tris-borate (pH 9.5)-10% methanol] in a semidry apparatus [9]. After the membrane was stained with Coomassie Blue, the visible 50 kDa (light chain) and 100 kDa (heavy chain) protein bands or appropriate peptide bands from proteolysis of each chain were cut out. The N-terminal amino acid sequences of each chain and peptide were determined by a gas phase protein sequencer (Model 470A; Applied Biosystems, Foster City, CA, U.S.A.) equipped with on-line PTH analysis using the regular program 03RPTH. The PTH-derivatives were analyzed by reverse-phase HPLC (Model 120A; Applied Biosystems, Foster City, CA, U.S.A.).

Purification of phage and extraction of DNA: Converting phage d-16ϕ was propagated with indicator D-16nt in TYG broth as described by Oguma *et al.* [17]. The phage particles were precipitated with 10% polyethyleneglycol 6000 and 0.5 M NaCl at 4°C overnight [30]. The precipitated phages were purified by the CsCl stepwise gradient procedure reported previously [24]. The purified phage particle was dialyzed against 10 mM Tris-HCl (pH 8.5)-10 mM EDTA buffer containing 50% formamide overnight, and then dialyzed against 50 mM Tris-HCl (pH 8.5)-0.1 M NaCl-10 mM EDTA buffer. The DNA was extracted by phenol treatment and was precipitated by adding 2.5-fold volume of icecold ethanol.

Cloning and selection of clones producing neurotoxin: The phage DNA was digested with *Eco*RI and the resulting fragments were ligated into the *Eco*RI site of λgt11. The phage packed *in vitro* with Packagene (Promega Biotec, Madison, WI, U.S.A.) was plated on *Escherichia coli* Y1090 (r−) with 10 mM isopropyl-B-D-thiogalacto-pyranoside (IPTG) and 0.2% X-Gal to give about 300 colorless plaques produced by recombinant phages. Nitrocellulose filters (BA-85; S & S, Dassel, Germany) were overlaid on agar plates and left in contact at 4°C overnight. The neurotoxin-producing clones were detected with goat anti-type D neurotoxin serum (Chiba Serum laboratory, Ichikawa, Japan) by using the Immuno-blot screening system (Bio Rad laboratory, Richmond, CA, U.S.A.).

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The isolated clones were amplified and subjected to characterization by a clonal epitope technique [29]. The expressed fusion proteins produced from 10,000 phage plaques of each clone in the presence of IPTG, were adsorbed to nitrocellulose filters. The filters were then incubated overnight with the polyclonal antiserum. Epitope-purified antibodies on each filter were eluted and used to detect the neurotoxin protein by Western blot analysis [28].

Southern hybridization: The oligonucleotide (5'-TTT AAT TAT AGT GAT CCT GTT-3') corresponding to Phe^{7} to Val^{13} of the N-terminal region of the light chain was synthesized by the phosphoamidite method using an automated DNA synthesizer (Model 380A: Applied Biosystems, Foster City, CA, U.S.A.), and used as a probe to identify botulinum neurotoxin type D-specific sequence. The oligonucleotide was labeled with [γ-^{32}P] ATP (Amersham Japan, Tokyo, Japan) and T4-polynucleotide kinase (Boehringer Mannheim, Mannheim, Germany).

Cloned DNA for the neurotoxin gene was completely digested with several restriction endonucleases (Toyobo, Osaka, Japan). Fragments were separated by electrophoresis on a 1% agarose gel and transferred to a nitrocellulose filter. *Hin*dIII-digested λphage DNA was also loaded on the agarose gel as a size marker.

Hybridization was performed with 10^{7} cpm of the labeled oligonucleotide probe at 40°C overnight as described by Maniatis *et al.* [14]. The filter was then exposed to Fuji RX film (Fuji Photo Film, Tokyo, Japan) overnight.

DNA sequencing: The cloned DNA was digested with *Hin*dIII or *Xba*I and the fragments were subcloned into appropriate sites of M13 mp18/19 or pUC 118/119 to provide templates. Large fragments were deleted using a Takara deletion kit (Takara Shuzo, Kyoto, Japan), which contained exonuclease III, mung bean nuclease and Klenow enzyme. DNA sequencing was performed by the dideoxy-chain termination method [21] with a [α-^{35}S] dCTP (Amersham Japan, Tokyo, Japan) and Sequenase™ DNA-sequencing kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Some regions were directly sequenced using appropriate synthetic oligonucleotide primers complementary to the neurotoxin gene with an AmpliTaq sequencing kit (Takara Shuzo, Kyoto, Japan). The Microgenie sequence analysis program from Beckman Instruments (Pal Alto, CA, U.S.A.) was used for the complete DNA sequence, comparing nucleic acid and protein sequences and protein hydrophobicity.

RESULTS

N-Terminal amino acid sequence of light and heavy chains of C. botulinum neurotoxin type D: Partial amino acid sequences of the light and heavy chains were determined by the direct microsequencing method. The amino acid sequence determined for the light chain, 1 through 20 residues, was Thr-Trp-Pro-Val-Lys-Asp-Phe-Asn-Tyr-Ser-Asp-Pro-Val-Asn-Asp-Asn-Asp-Ile-Leu-Tyr (Fig. 1). The heavy chain from the purified neurotoxin preparation by electro-blotting after SDS-PAGE, whose sequence was Asn-Ser-Arg-Asp-Asp-Ser-Thr-Cys-Ile-Lys-Val-Lys-Asn-Asn-Arg-Leu-Pro-Tyr-Val-Ala (Fig. 1), was also isolated.

To clarify the deduced amino acid sequence of the neurotoxin, several peptides were prepared by limited proteolysis with V8 protease and lysyl endopeptidase which cleave at the COOH-terminal side of Asp or Glu, and Lys residues, respectively. Five peptides were obtained from major bands generated by the digestion of the neurotoxin during the 2nd electrophoresis. The sequences and locations of each peptide on the neurotoxin molecule are illustrated in Fig. 1.

Cloning and isolation of the Type D neurotoxin gene: Purified d-16ϕ DNA was digested with *Eco*RI and the resulted fragments were ligated to λgt11 and packed in the phage particles. The library was plated with *E. coli* Y1090. The initial screening gave 4 positive plaques which were subjected to secondary

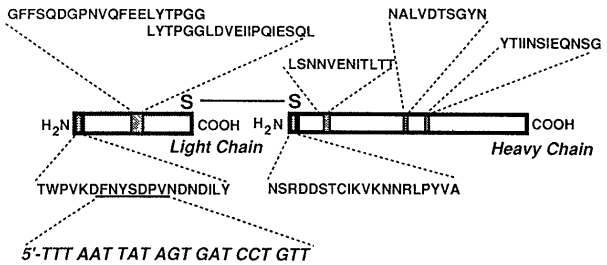


Fig. 1. Schematic structural model of botulinum type D neurotoxin and N-terminal amino acid sequence of light and heavy chains, and peptides prepared by limited proteolysis with V8 protease and lysyl endopeptidase. A synthetic oligonucleotide corresponding to amino acid sequence 7–13 of the light chain was used to identify the gene encoding botulinum type D neurotoxin.

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screening. One of these plaques which was designated as D-3 with an insertion of about 10 kbp DNA was characterized by the clonal epitope selection method. Epitope specific antibody against the expressed fusion protein from D-3 recognized the purified botulinum type D neurotoxin in protein blot analysis. This suggests that D-3 DNA encodes a protein that contains the antigenic determinant of botulinum type D neurotoxin.

Fragments in *Hin*dIII and *Xba*I digestion were transferred to a nitrocellulose filter and hybridized with synthetic oligonucleotide probe (21mers). Southern blot analysis revealed that a 2 kbp fragment in *Hin*dIII digestion and a 7.35 kbp fragment in *Xba*I digestion were identified with probes as shown in Fig. 2. Thus, the nucleotide sequence encoding the N-terminus of the neurotoxin was located on the 2 kbp *Hin*dIII fragment of D-3 insert DNA (Fig. 2).

Nucleotide and deduced amino acid sequence of the DNA for neurotoxin type D: The DNA fragments containing the type D neurotoxin gene were sequenced from restriction sites or specific sites, and on both DNA strands following the strategy outlined in Fig. 3. The resulting nucleotide sequence (4,089 nucleotides) included the complete coding region and parts of the 5'- and 3'-end noncoding regions. The DNA sequence and the deduced amino acid sequence of botulinum neurotoxin type D are presented in Fig. 4. They contained a single open reading frame coding for 1,275 amino acid residues, corresponding to a polypeptide with a calculated molecular weight of 146,785. The coding sequence begins with a codon ATG for Met and the second codon specifies Thr, the N-terminal residue of the light chain of the neurotoxin type D as determined in this study. The deduced structure also contains the cleavage site between Lys^{442} and Asn^{443}, which indicates the start of the heavy chain in agreement with the N-terminal amino acid sequence of the heavy chain. Furthermore, the deduced structure also contains sequences for peptides prepared by V8 protease or lysyl endopeptidase digestion of the light and heavy chains of the neurotoxin protein (dot-underlined in Fig. 4).

The neurotoxin gene had an [A+T] content of 74.3% with the coding region and average of 75.7% with 5'- and 3'-end noncoding regions. The findings are in agreement with the previously determined [A+T] content of phage d-16ϕ DNA [24] and other clostridial DNAs [15].

The 5'-end untranslated region was analyzed for the presence of a regulatory sequence. A Shine-Dalgarno [22] consensus sequence [AGGAGA] was found 16 nucleotides upstream from the initiation ATG codon as underlined in Fig. 4. A Pribnow sequence (−10 region) was present 86 nucleotides upstream from the translation start codon, although no corresponding −35 region was detected. Two inverted repeated structures were located immediately downstream from the termination codon (Fig. 4). However it is not clear whether these sequences function as terminators, because the

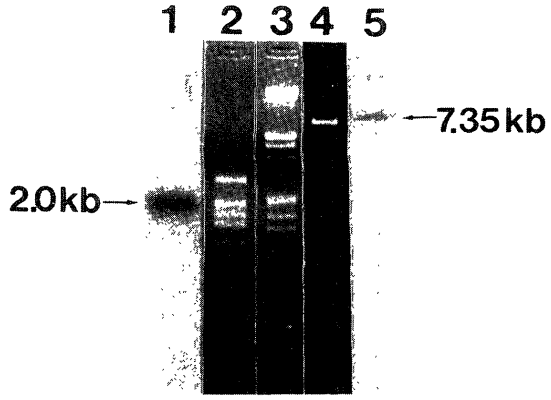


Fig. 2. Southern hybridization analysis. Inserted DNA of clone D-3 DNA (10 μg) was digested with restriction enzymes, *Hin*dIII (lane 1, 2) and *Xba*I (lane 4, 5), followed by separation by electrophoresis on a 1% agarose gel with *Hin*dIII-digested DNA as a size marker (lane 3). The fragments for *Hin*dIII (lane 1) and *Xba*I (lane 5) digests were transferred to a nitrocellulose filter and hybridized with 5'-labeled oligonucleotide probe, under low stringency conditions as described in "MATERIALS AND METHODS".

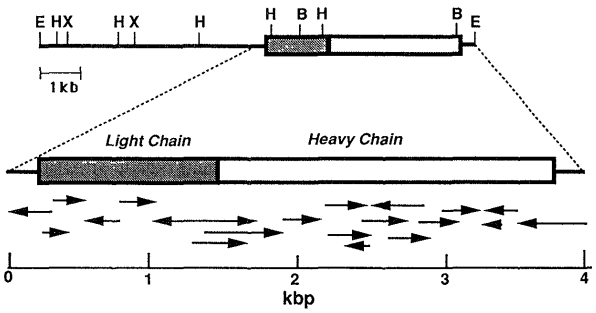
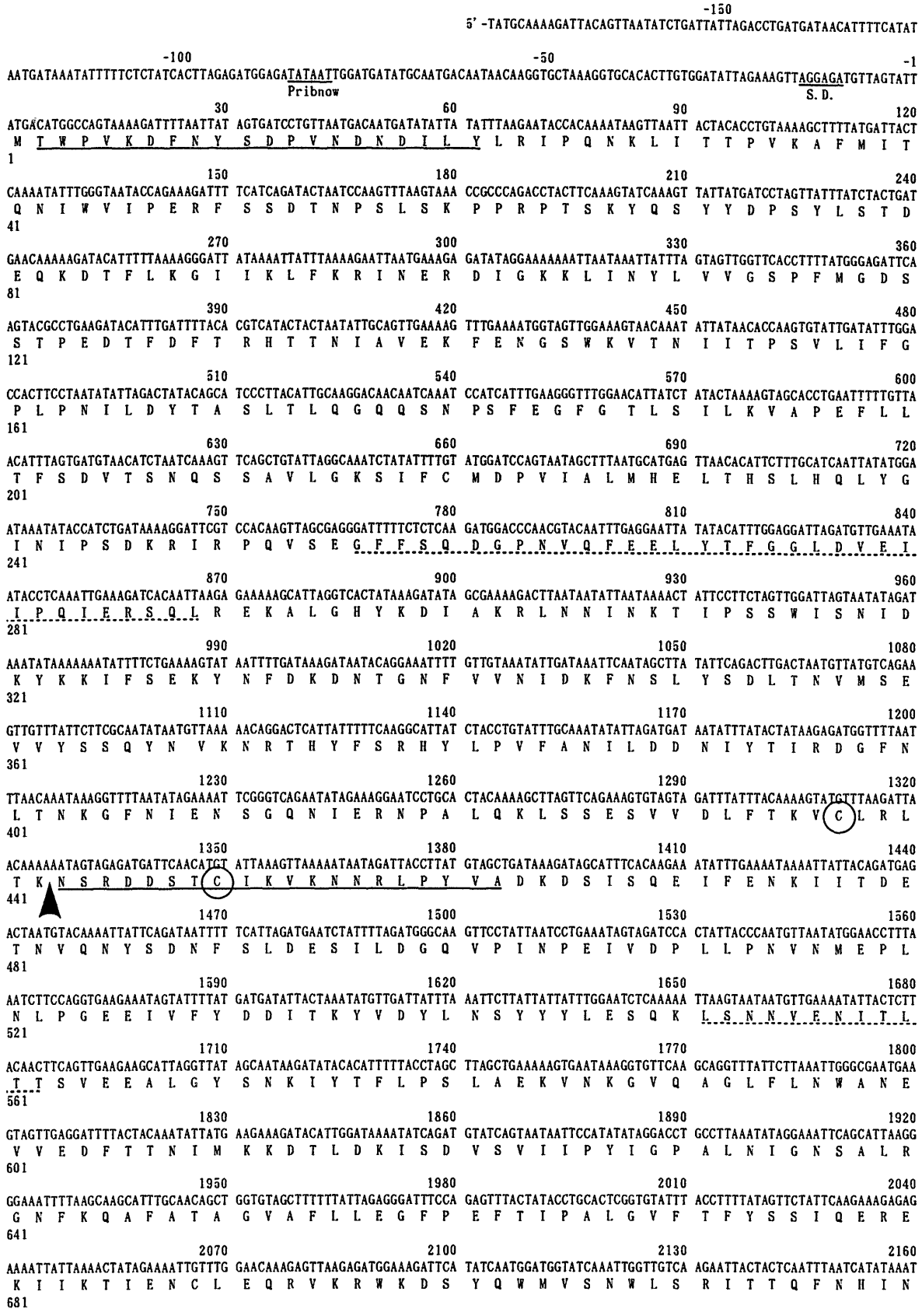


Fig. 3. Restriction map of insertion of D-3 DNA and sequencing strategy of the region containing the type D neurotoxin gene. The abbreviations for restriction enzymes; E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; X, *Xba*I. Coding region for the type D neurotoxin are shown by boxes. Arrows show the direction of sequencing and are aligned in the 5' to 3' direction.

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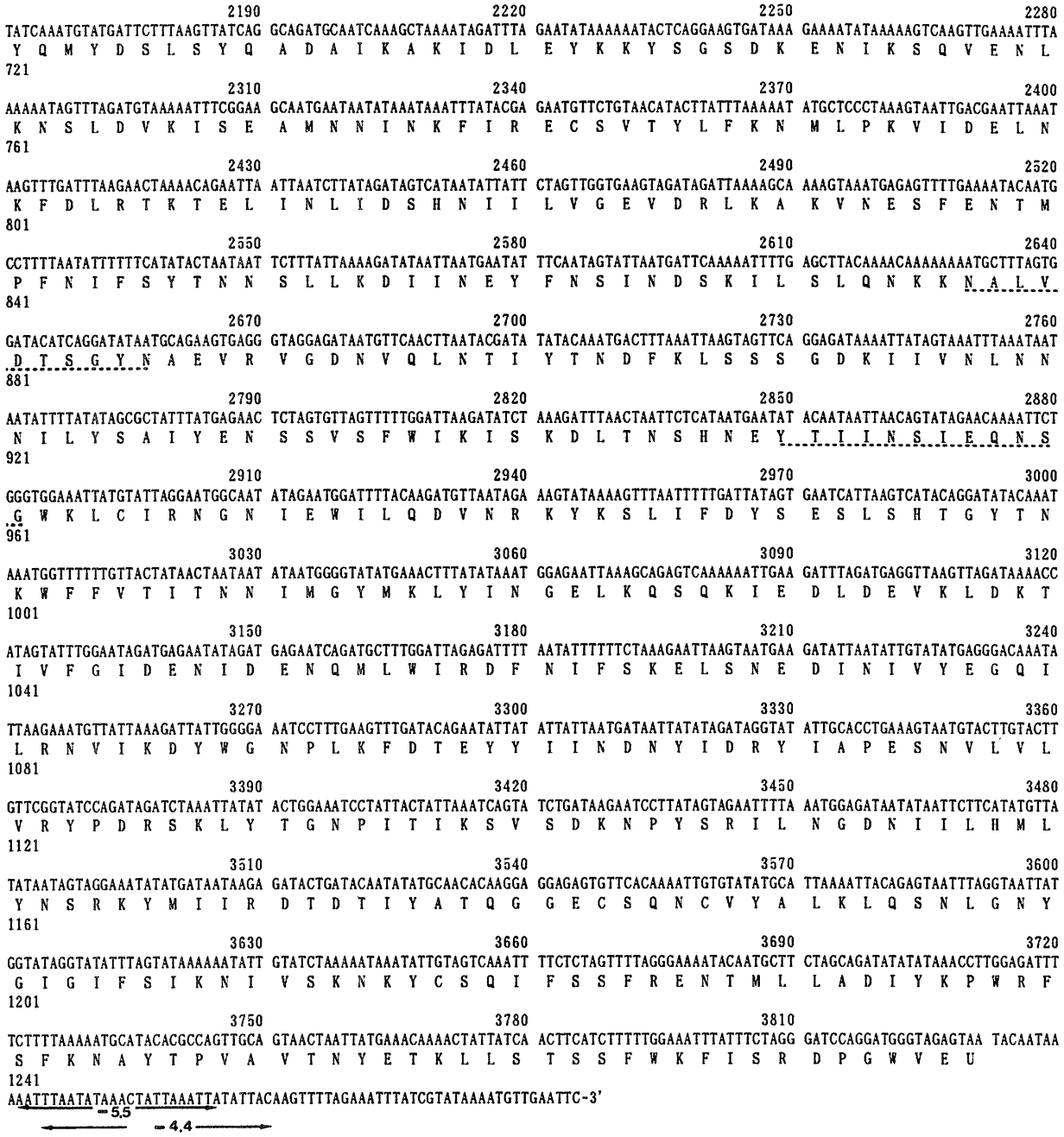


Fig. 4. Nucleotide sequence of the botulinum type D neurotoxin gene and deduced amino acid sequence. Nucleotides are numbered in the 5' to 3' direction, beginning with the first residue of ATG triplet encoding the initiator methionin. Nucleotides on the 5'-side of residue 1 are given negative numbers. The putative Shine-Dalgarno sequence is indicated by the underlined region. Two inverted repeat structures are underlined with divergent arrows. Numbers represent free energy values in Kcal/mol. The arrow head between Lys^{442} and Asn^{443} indicates the start of the heavy chain in agreement with the data obtained in this work. Circled Cys^{437} and Cys^{450} represent putative residues involving in a disulfide bond between the light and heavy chains in the nicked neurotoxin molecule. Amino acid residues determined by protein sequence analysis of the peptides from limited proteolysis of the neurotoxin protein with V8 protease and lysyl endopeptidase are dot-underlined.

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calculated free energy, −5.5 Kcal and −4.4 Kcal, is insufficient to form a stem-loop structure.

DISCUSSION

The complete amino acid sequence of botulinum type D neurotoxin is presented here. This sequence was deduced from the complete nucleotide sequence of the gene, and partial amino acid sequences of the neurotoxin protein were determined by the direct microsequencing method. The deduced amino acid sequence corresponds to the N-terminal amino acid sequences of the light and heavy chains of type D neurotoxin and five polypeptides obtained from the neurotoxin after limited proteolysis.

While this work was in progress, Binz *et al.* [1] reported the nucleotide sequence of the gene encoding botulinum type D neurotoxin. For the most part, their predicted nucleotide sequence corresponds with that determined in this study. However a few discrepancies exist in the nucleotide sequence for the coding region of the type D neurotoxin gene. The resulting substitution of a codon led to the alteration of the amino acid residues, Asn^{489}, Lys^{646} and Arg^{1122} in our sequence to Lys, Asn and Glu, respectively. Botulinum type D neurotoxins have been shown to have a mosaic structure consisting of strain-specific epitopes and epitopes common to toxin type. Binz *et al.* [1] did not indicate the strain employed in their experiment, so it is unclear as to whether the difference in the three nucleotides is due to the different strains employed in Binz *et al.*'s, and the present experiments.

A comparative alignment of the sequence established in this study with the sequences of other types of botulinum type C_1 [11] and A [2, 27] neurotoxins and tetanus neurotoxin [4, 7] revealed that type D neurotoxin exhibited 52.2% identity with type C_1, 33.2% with type A, and 32.3% with tetanus neurotoxin, respectively. It is apparent that type D neurotoxin exhibits a higher degree of identity to the type C_1 neurotoxin than to other neurotoxins. This is to be expected since *C. botulinum* type D and C_1 are mutually convertible by specific bacteriophages [6, 10], and type D and C_1 neurotoxins cross-reacted antigenically [18–20]. Also, the pattern of alignment displayed in the dot-matrix plot (Fig. 5) indicates that the heavy chains exhibit a higher degree of alignment than the light chains; heavy chain 56.0%, light chain 46.7%. Strikingly homologous regions were also found in the heavy chains. The sequence from Lys^{522} to Asn^{898} of the type D neurotoxin is essentially the same as that from Lys^{526} to Asn^{902} of the type C_1 neurotoxin (80.7% identity).

A hydrophobicity analysis according to the method of Kyte and Doolittle [12] indicated that two markedly hydrophobic regions are distributed on the neurotoxin molecule as shown in Fig. 6. The first of these predicted regions contained about one hundred amino acid residues composed of hydrophobic and uncharged amino acids from Trp^{146} to Ile^{243} on the light chain. In this region, the His residue rich sequence, extending from Leu^{227} to Asn^{242}, was highly conserved among botulinum type A [2, 27] and C_1 [11], and tetanus [4, 7] neurotoxins. The second region was located on the heavy chain from Val^{621} to Lys^{681}, and this region was also conserved in botulinum type A [2, 27] and C_1 [11], and tetanus [4, 7] neurotoxins. These two domains have sufficient length and hydrophobicity to be able to potentially span the mammalian cell membrane as reported in tetanus [4] and botulinum type A [2] neurotoxins.

Botulinum neurotoxins are synthesized intracellularly as a single polypeptide chain. They are activated by proteases in the culture or mammalian stomach and are cleaved to form a dichain consisting of a light chain and a heavy chain linked by a

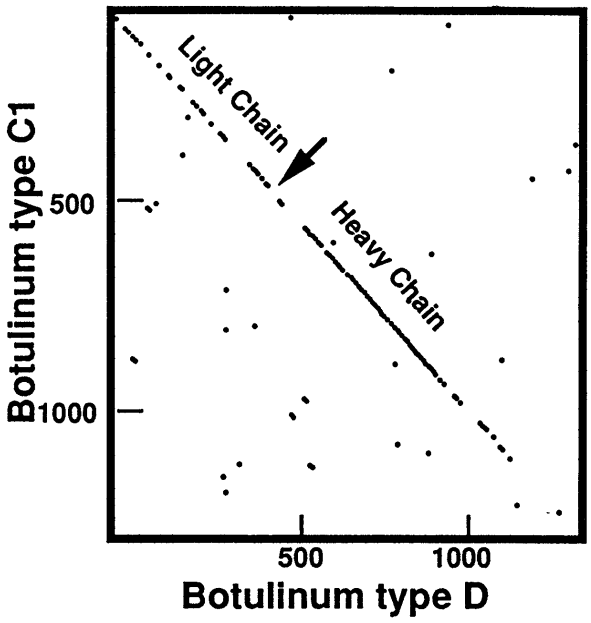


Fig. 5. Dot matrix plot of botulinum type D and C1 neurotoxin similarity. Similarity parameters show a 75% match with a window size of 8 amino acids.

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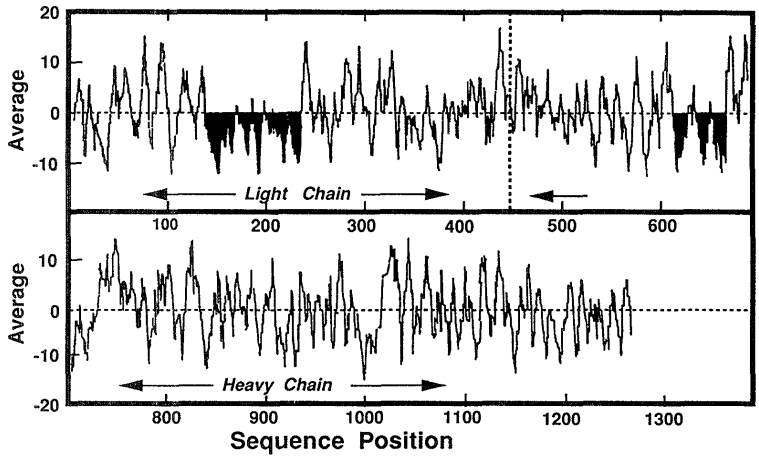


Fig. 6. Hydrophobicity of the botulinum type D neurotoxin as a function of sequence position. A running 11-residues average was used. Values were calculated by the method from ref. 12.

disulphide bond. The mechanism of activation (nicking) however has been unclear. Recently Moriishi *et al.* [16] reported N-terminal amino acid sequences of botulinum type D neurotoxins, however they were incomplete. In this paper, the complete N-terminal amino acid sequence (20 amino acid residues) of the heavy chain of botulinum type D neurotoxin is reported for the first time. The nicking site of the type D neurotoxin is also indicated. However the notion that primary cleavage occurs at a different peptide bond and that the N-terminal residue, Asn, is the result of the subsequent action of proteases cannot be discounted.

The availability of the entire sequence of botulinum type D neurotoxin arising from this work will allow a detailed study of the neurotoxin and may lead to a fuller understanding of the mode of action of the botulinum neurotoxin at the molecular level. Expression studies at the analytical level may help to define the epitopes and functional domain of the botulinum neurotoxin molecule and provide the toxoid for the formulation of an improved vaccine for botulism in man and animals.

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