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Isolation, Purification, and Characterization of Fragment B, the NH_2-Terminal Half of the Heavy Chain of Tetanus Toxin

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Fragment B, the N-terminal half of the heavy chain, an important domain of the tetanus neurotoxin molecule, was isolated for the first time. Tetanus toxin (composed of three domains, A, B, and C) was prepared from culture filtrates. Fragment A-B, derived from the toxin treated mildly with papain, was used for the isolation of fragment B. Fragment A-B obtained was dissociated into fragments A and B by reduction with 100 mM dithiothreitol and treatment with 2 M urea. Fragment B was separated from fragment A by ion-exchange column chromatography on a Mono Q column equilibrated with 20 mM Tris hydrochloride buffer (pH 7.6), containing 1 mM dithiothreitol and 2 M urea, in a fast-protein liquid chromatography system by elution with a linear gradient of 0 to 0.5 M NaCl. Fragment B was obtained in two forms having molecular weights of 48,000 ± 2,000, which were indistinguishable by sodium dodecyl sulfate-gel electrophoresis or antigenic specificity, but distinguishable on polyacrylamide gel electrophoresis without sodium dodecyl sulfate and on isoelectric focusing (pI 6.7 and 7.3). The recovery of fragment B was 50 to 72% of that of fragment A-B on a molar basis. Purified fragment B was not toxic to mice on intravenous or intramuscular injection at doses of up to 100 μg, but was found to form channels (ca. 2.3 pS) in a lipid bilayer membrane by a patch clamp technique. The role of domain B of the tetanus toxin molecule in the mechanism of action of the toxin is discussed.

Tetanus toxin is one of the most poisonous substances known (39). The toxin, when injected intramuscularly at low doses, is taken up at the motor endplates and carried by retrograde axonal transport to the central nervous system, where it first blocks inhibitory synapses and then excitatory synapses (36, 37), producing delayed spastic paralysis typical of tetanus, and finally contracture. At enormous doses, the toxin blocks peripheral neuromuscular transmission, producing the acute botulinumlike flaccid paralysis (22, 23, 34) observed in particular experimental conditions and in some cases of severe clinical tetanus. Recent findings have indicated that the common underlying mechanism of these symptoms is inhibition of release of inhibitory and excitatory neurotransmitters by the toxin at presynaptic sites of the nerve terminals (12). However, the exact structure-function relationship in the tetanus toxin molecule is unknown.

The toxin (molecular weight [mol wt], ca. 150,000) is composed of three domains (20, 21, 26), A, B, and C, according to a new nomenclature (8th International Conference on Tetanus, Leningrad, 1987), each with a mol wt of ca. 50,000 (Fig. 1). Two complementary nontoxic fragments, the light chain, fragment A (mol wt, 52,000), and the heavy chain, fragment B · C (mol wt, 98,000) (Fig. 1), were isolated from extracellular, nicked toxin in sufficiently native forms to be reconstituted into a whole toxin (24, 25). Another set of two fragments, fragment A-B and fragment C (mol wt, 52,000) (Fig. 1), were obtained by dissociating the toxin by mild treatment with papain (2, 14, 26). Fragment C is nontoxic and is known to bind gangliosides, putative tetanus toxin binding substances at motor endplates of animals, and synaptic membranes and also to function in carrying the toxin from the peripheral sites by retrograde axonal transport to the central nervous system (2–4, 15). In contrast to these isolated, nontoxic fragments (fragments A, B · C, and C), fragment A-B, which constitutes the NH_2-terminal two-thirds of the toxin molecule, showed nonspastic toxicity (6, 11, 13; K. Ozutsumi, D.-L. Lei, N. Sugimoto, and M. Matsuda, Toxicon, in press), inducing "weakness" (Ozutsumi et al., in press). Furthermore, fragment A-B has been reported to inhibit secretion or release of catecholamine when it was introduced into chromaffin cells (1, 30) and to block synaptic transmissions when it was injected intraspinally into cats (38). Therefore, fragment A-B appears to play an important role for the toxin in eliciting toxicity at direct target sites of sensitive cells.

As for the roles of the domains of fragment A-B in the action of tetanus toxin, domain B of the toxin molecules was recently concluded, from indirect studies on the neutralizing effect of Fab fragments of polyclonal or monoclonal antibodies directed to the region of domain B in the tetanus toxin molecule, to be critical for expression of the toxin action (5). For direct studies on the function of fragment B, its isolation is essential, but it has not previously been isolated (5). This paper reports the isolation of fragment B of the tetanus toxin molecule from fragment A-B and its purification and characterization. The role of domain B of the toxin in the mechanism of action of tetanus toxin is also discussed.

MATERIALS AND METHODS

Bacterial strain and preparation of tetanus toxin. A Biken substrain of the Harvard A47 strain of *Clostridium tetani* was used for toxin production. Extracellular toxin was prepared from culture filtrates after incubation of the organisms in modified Latham medium at 35°C for 5 days as described previously (24). The toxin was isolated and purified by gel permeation chromatography as described previously (24, 28).

Fragment A-B. The purified tetanus toxin was subjected to

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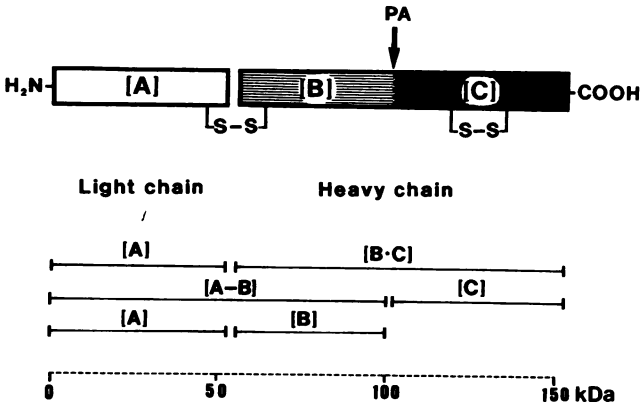

FIG. 1. Two-chain model of tetanus toxin composed of three domains, A, B, and C (tripartite model), according to a new nomenclature, and the fragments of the toxin. Tetanus toxin prepared from bacterial cells is a single polypeptide chain (mol wt, ca. 150,000). The toxin prepared from the culture filtrate (extracellular toxin) is a nicked form, two-chain molecule consisting of a light chain (fragment A; mol wt, ca. 52,000) and a heavy chain (fragment B · C; mol wt, ca. 98,000) linked by a disulfide bridge and noncovalent bonds. Mild treatment of the toxin with papain cleaved the toxin at the site indicated by an arrow (PA) into two components, fragment A-B (mol wt, ca. 99,000) and fragment C (mol wt, ca. 52,000).

mild treatment with papain to dissociate it into fragments A-B and C as described previously (26, 29; Ozutsumi et al., in press). Fragment A-B was then separated and purified from the digest by gel permeation chromatography on a TSK G3000SW column (Toyo Soda Co., Tokyo, Japan) equilibrated with 0.1 M NaK phosphate buffer (pH 6.8) in a high-performance liquid chromatography system (Toyo Soda Co.) as described previously (29; Ozutsumi et al., in press).

Thiol reduction and urea treatment of fragment A-B. Fragment A-B (0.5 to 1.2 mg of protein per ml in 0.1 M NaK phosphate buffer [pH 6.8]) was reduced with dithiothreitol (DTT; final concentration, 100 mM) at 25°C for 60 min. The reduced fragment A-B was treated with solid urea at various concentrations (1 to 8 M) for urea-polyacrylamide gel electrophoresis or at a final concentration of 2 M for ion-exchange chromatography.

Polyacrylamide gel electrophoresis. Conventional polyacrylamide gel electrophoresis in 5% gel (column) or in 7% gel (slab) and urea-polyacrylamide gel electrophoresis in 5% gel (column) were carried out in 10 mM Tris–77 mM glycine buffer (pH 8.6) as the electrode buffer by the methods originally described by Davis (9) and Jovin et al. (18), respectively. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in 8 to 25% gradient gel and isoelectric focusing were carried out in a Phast System (Pharmacia LKB, Uppsala, Sweden).

Separation of fragment B from reduced, urea-treated fragment A-B. The reduced, urea-treated fragment A-B (0.5 to 1.2 mg of protein per ml; 2.2 ml) was applied to a prepacked Sephadex G-25 column (PD-10; Pharmacia LKB) equilibrated with 20 mM Tris hydrochloride buffer (pH 7.6) containing 1 mM DTT and 2 M urea (buffer A), eluted with 3.5 ml of buffer A, and filtered through a membrane filter (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.) with a pore size of 0.2 μm (final volume, ca. 4 ml). Ion-exchange chromatography was carried out in a fast-protein liquid chromatography system (Pharmacia LKB) composed of two model P-500 high-precision pumps, a gradient programmer (model GP-250), and a single-path UV monitor, model UV-1 (Pharmacia LKB). The filtered sample (ca. 4 ml, 1.1 to 4 mg of protein) was applied on a prepacked column of Mono Q HR5/5 (Pharmacia LKB) equilibrated with buffer A. Material was eluted with a linear gradient of NaCl (0.025 M NaCl increase per min) formed using buffers A and B (buffer B is buffer A supplemented with 0.5 M NaCl) at a flow rate of 1 ml/min. The protein content of the eluate was monitored at 280 nm, and each peak was collected.

Immunodiffusion test. The double-diffusion precipitation method originally described by Ouchterlony (27) was employed using 1% agarose containing 50 mM Tris–0.6 M glycine buffer (pH 8.5) and 1 mM EDTA.

Toxicity test. Toxicity was examined in OF1 mice of both sexes, weighing 20 to 25 g, by intravenous injection (0.1 ml) or intramuscular injection (0.1 to 0.5 ml) of samples.

Protein determination. Protein was measured by the method of Lowry et al. (19).

Fragment A and fragment B · C. Complementary fragments of tetanus toxin, fragment A and fragment B · C, were isolated and purified from the reduced, urea-treated extracellular toxin by gel permeation chromatography on an Ultrogel AcA (Pharmacia LKB) column as described previously (24).

Tetanus antitoxin serum. Horse antitoxin serum (lot no. 0044) was a gift from the National Institute of Health, Tokyo, Japan.

Chemicals. DTT was obtained from Nakarai Tesque Co., Kyoto, Japan; urea (for biochemistry) was obtained from E. Merck, Darmstadt, Federal Republic of Germany; and asolectin (soybean lecithin) was obtained from Daigo Eiyo Kagaku Co., Osaka.

Detection of channel formation in lipid biolayers by a patch clamp technique. Asolectin was suspended at a concentration of 9 mg/ml in 10 mM potassium acetate buffer (pH 4.0) containing 150 mM KCl by sonication under continuous bubbling with N_2 gas. Two hundred microliters of the suspension was mixed with an equal volume of solution of tetanus toxin (100 μg/ml), fragment A-B (100 μg/ml) or fragment B (50 μg/ml) in the same buffer and sonicated at 37°C for 2 min. Test mixtures were prepared by adding 100 μl of the resulting sonically treated material extract to 0.4 ml of the same buffer. The formation of lipid monolayers on the aqueous surface of the test mixture was confirmed by observing drops of water running over the surface. Patch capillary pipettes were filled with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.0) containing 150 mM KCl. Patch clamping was performed as described previously (35) on a lipid bilayer formed at the tip of the patch pipettes (electrical resistance, 7 to 30 MΩ) by the method of Suarez-Isla et al. (33), namely, dipping the tip into the test mixture twice and then clamping the voltage of the inside of the patch pipette against the bath electrode and amplifying the current passed through the lipid bilayer at the tip of the patch pipette.

Determination of NH_2-terminal sequence. The NH_2-terminal sequence of the fragments of the toxin was analyzed on electroblotted proteins by the microsequence method of Hirano (16).

RESULTS

Dissociation of fragment A-B by reduction with DTT and treatment with urea. To determine the optimal conditions for dissociation of fragment A-B, we first treated fragment A-B

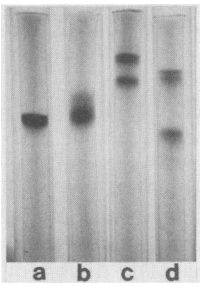

FIG. 2. Conventional (lane a) and urea-polyacrylamide gel (lanes b through d) electrophoresis. Lane a, Untreated fragment A-B; lane b, 2 M urea-treated fragment A-B in 2 M urea gel; lane c, reduced and 4 M urea-treated fragment A-B in 2 M urea gel; lane d, reduced and 2 M urea-treated fragment A-B in 2 M urea gel. Approximately 15 μg of protein was applied to each gel (5% gel). Electrophoresis was carried out at a constant current of 2 mA per gel at 4°C for 120 min. Migration was from top to bottom.

(0.5 mg/ml in NaK phosphate buffer [pH 6.8]) with 100 mM DTT at 25°C for 60 min and then treated it with various concentrations of urea (0 to 8 M) and subjected the preparation to electrophoresis in the presence of various concentrations (0 to 8 M) of urea. Treatment of the reduced fragment A-B with 2 M urea and subsequent electrophoresis in the presence of 2 M urea gave the best separation of components (Fig. 2). Thus the components in fragment A-B are linked by a disulfide bridge and noncovalent bonds that can be dissociated with 2 M urea.

Separation of the components derived from the reduced and urea-treated fragment A-B by fast-protein liquid chromatography on a Mono Q column. Fragment A-B was dissociated into its components under the optimal conditions for dissociation described above (reduction with 100 mM DTT and treatment with 2 M urea) and then subjected to ion-exchange chromatography on a Mono Q column in the presence of 1 mM DTT and 2 M urea. Figure 3 shows the elution profile of the reduced, urea-treated fragment A-B with a linear gradient of 0 to 0.5 M NaCl. Three sharp peaks (designated as fractions I, II, and III in order of their elution) were obtained at 0.10, 0.13, and 0.19 M NaCl (Fig. 3). The ratio of these peaks was approximately 1:1:2 on a protein basis.

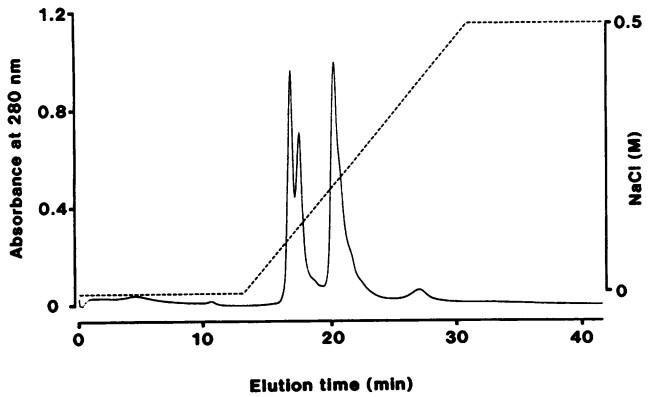

FIG. 3. Elution profile (fast-protein liquid chromatography) of DTT-reduced and urea-treated fragment A-B on a Mono Q column. Buffers as described in Materials and Methods. The broken line indicates the gradient from 0 to 0.5 M NaCl, obtained with buffers A and B. Flow rate, 1 ml/min. Sample: Volume, 4 ml; amount, 4 mg of protein.

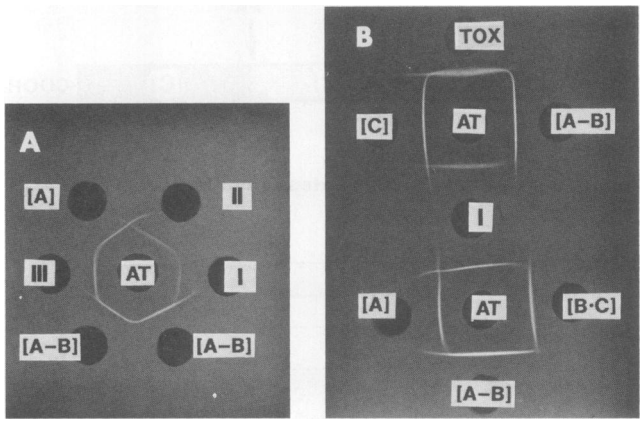

FIG. 4. Immunodiffusion patterns of fragment A-B, fractions I, II, and III, fragments A, B · C, and C, and toxin against horse antitoxin. Panel A: [A], fragment A (0.5 mg/ml); [A-B], fragment A-B (0.5 mg/ml); I, fraction I (0.5 mg/ml); II, fraction II (0.5 mg/ml); III, fraction III (0.5 mg/ml); and AT, antitoxin (1,000 U/ml). Panel B: [A], fragment A (0.5 mg/ml); [A-B], fragment A-B (0.5 mg/ml); [B · C], fragment B · C (0.5 mg/ml); [C], fragment C (0.5 mg/ml); and TOX, toxin (1 mg/ml).

Antigenic specificities of the purified components derived from reduced, urea-treated fragment A-B. Figure 4 shows the antigenic relationship of fragment A-B and the components (fractions I, II, and III) derived from the dissociated fragment A-B. The antigenic specificities of fractions I and II were identical, while that of fraction III was different from these two fractions but identical to that of fragment A (Fig. 4A). These components all showed partial antigenic identity with fragment A-B (Fig. 4A). Figure 4B shows the results of antigenic analyses of fraction I, using purified fragments A, B · C, and C: fraction I showed partial antigenic identities with fragment A-B and fragment B · C, but differed in antigenic specificity from fragment A and fragment C. The same pattern of antigenic specificity as that of fraction I (Fig. 4B) was observed with fraction II. Therefore we concluded that fractions I and II had the same antigenic specificity and that they were antigenically identical to domain B of tetanus toxin, while fraction III had the same antigenicity as domain A of the toxin.

Properties of the purified components. Figure 5 shows the electrophoretic patterns of the toxin, fragment A-B, and fractions I, II, and III in polyacrylamide gel in the presence (Fig. 5A) and absence (Fig. 5B) of SDS. Fractions I and II gave single protein bands with the same mol wt of 48,000 ± 2,000 on SDS-gel electrophoresis (Fig. 5A), but single bands with different electrophoretic mobilities in gel without SDS (Fig. 5B). On isoelectric focusing, fractions I and II showed pI values of 7.3 and 6.7, respectively. The mol wt of fraction III isolated from fragment A-B was 54,000 ± 2,000 on SDS-gel electrophoresis. From their antigenic specificities and mol wts and the difference in their electrophoretic mobilities, we concluded that fractions I and II corresponded to domain B of fragment A-B with microheterogeneity in molecular structure. Therefore, we named fractions I and II fragments B_1 and B_2, respectively. The recovery of fragment B (fragments B_1 plus B_2) was 49.5 to 72% of that of fragment A-B on a protein basis. Preliminary analysis of the NH_2-terminal sequence of fragment B_2 gave a single amino terminus, aspartic acid, confirming that the preparation was highly purified. The NH_2-terminal sequence of fragment B_2

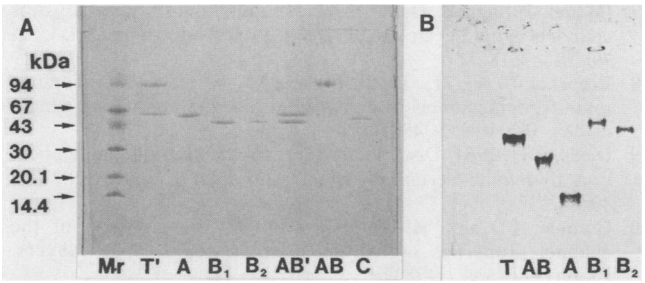

FIG. 5. Polyacrylamide gel electrophoresis of toxin, fraction I, fraction II, and fraction III in the presence (A) and absence (B) of SDS. Panel A, SDS-polyacrylamide 8 to 25% gradient gel: Mr, protein markers for mol wt; T', reduced toxin; A, fraction III; B_1, fraction I; B_2, fraction II; AB', reduced fragment A-B; AB, untreated fragment A-B; C, fragment C. Samples of approximately 10 μg of protein for lane Mr, 2 μg of protein each for lanes T', AB', and AB, and 1 μg of protein each for lanes A, B_1, B_2, and C were applied. Electrophoresis was carried out in a Phast System at a constant current of 10 mA at room temperature for 1 h. Panel B, 7% polyacrylamide gel without SDS: T, untreated toxin; AB, untreated fragment A-B; A, fraction III; B_1, fraction I; B_2, fraction II. Samples of approximately 5 μg of protein for lane T, 2 μg of protein for lane AB, and 1 μg of protein each for lanes A, B_1, and B_2 were applied. Electrophoresis was carried out at a constant current of 10 mA for 3 h. Migration was from top to bottom. Gels were stained with Coomassie brilliant blue R.

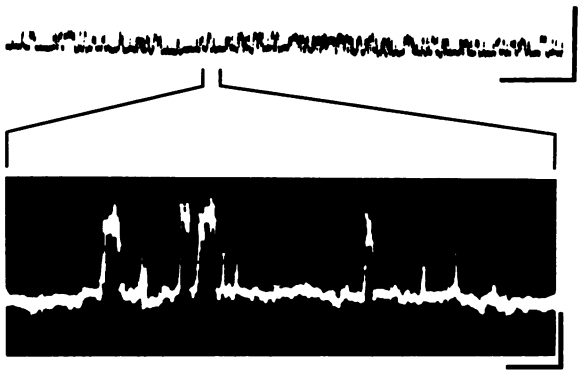

FIG. 6. Record of the membrane current obtained from an asolectin bilayer patch with incorporated fragment B preparation (fragment B_1) of tetanus toxin. A patch pipette with an electrical resistance of 15 MΩ was used. The asolectin bilayer patch formed at the tip of the pipette showed a resistance of 12.5 GΩ. The recording was made at a holding potential of 100 mV. The upper trace was a record with a high-cut filter of 1 kHz on paper. The lower trace is a photograph taken directly from the cathode ray oscilloscope. Calibration bars indicate 1.5 pA (ordinate) and 3 s (abscissa) in the upper trace and 0.2 pA (ordinate) and 150 ms (abscissa) in the lower trace.

was D-L-G-G-E-L-X(C)-I-K-I-K-N-E-D-L, indicating the fragment begins with Asp-461 in the tetanus toxin molecule.

Toxicity of the purified preparations of fragment B. When injected into mice intravenously or intramuscularly at doses of up to 100 μg of protein, fragments B_1 and B_2 did not produce symptoms of spastic paralysis typical of tetanus, botulinumlike flaccid paralysis (23, 34), which is observable on injection of an enormous dose of tetanus toxin, or weakness (13, 29; Ozutsumi et al., in press), as observed on injection of fragment A-B.

Fragment B forms channel in a lipid bilayer. Using the patch clamp technique, we examined the channel activities of asolectin bilayer membranes with incorporated fragment B by recording their membrane currents. Definite channel activities were observed in 19 patches out of 146 trials in the lipid membranes with incorporated fragment B in the presence of a pH gradient (pH 7.0 inside the patch capillary pipette and pH 4.0 outside), as in the case of lipid bilayers with incorporated toxin or fragment A-B.

Figure 6 shows an example of the current in a membrane with incorporated fragment B_1. The level of the membrane current shifted abruptly, indicating gating of the ion-permeable channels in the lipid bilayer patch. The channel activities in the membranes with incorporated fragment B (fragment B_1 or B_2) showed single conductance levels of 2.3 ± 0.2 pS.

DISCUSSION

In the present study we isolated fragment B for the first time. Dissociation of whole toxin into light chain (fragment A) and heavy chain (fragment B · C) requires treatment with 4 M urea and reduction with 100 mM DTT (24). In contrast, we found that a lower concentration of urea (2 M) gave the best separation of fragment B from fragment A (Fig. 2). Fragments B and A could then be separated from each other on an ion-exchange column because of their significant difference in pIs. Recently it was found that fragment A-B, from which fragment B was isolated, is toxic (11, 13, 29; Ozutsumi et al., in press) and blocks synaptic transmissions (38). We confirmed a peculiar, delayed nonspastic toxicity of fragment A-B in mice, which we termed weakness (Ozutsumi et al., in press), and found further that fragment A-B blocked both inhibitory and excitatory synapses simultaneously in the central nervous system when injected directly intraspinally into cats (38). Furthermore, fragment A-B was reported to inhibit exocytosis in adrenal chromaffin cells under particular conditions (1, 30). Therefore, fragment A-B appears to play an important role in the action of tetanus toxin.

To define in more detail the region of the toxin molecular responsible for its toxic effects, recently Bizzini et al. (5) examined the effects of masking various domains of the toxin with Fab fragments of antibodies and concluded that the area corresponding to fragment B is critical for expression of the toxicity of tetanus toxin. However, they could not examine the toxic effect of isolated fragment B as they were unable to isolate it (5). In this study we showed that the isolated fragment B did not show any toxicity in mice even at enormous doses of up to 100 μg. This is contrary to the conclusion of Bizzini et al. (5) that the toxic effect is associated only with fragment B. Cooperation of fragment B with fragment A is probably required to elicit toxic effects in vivo.

Tetanus toxin forms transmembrane ion channels in lipid bilayers (7, 8, 10, 17). Fragment B · C (17) and fragment A-B (10, 17, 32) of the toxin form ion channels with similar conductances. Since neither fragment A (17) nor fragment C (10) increases the ionic conductance of planar bilayers, it has been suggested that the channel-forming domain of the tetanus toxin molecule must be located in the domain corresponding to fragment B (10, 17). The present study gave, for the first time, direct evidence that fragment B forms ion channels in a lipid bilayer. Gamble and Montal (10) attempted to observe the channel-forming activity of domain B by adding reduced fragment A-B to lipid bilayer. However, under their conditions, fragment A-B had been reoxidized, or, even though it was reduced, fragments A and B were probably still united by noncovalent bonds as shown

above (Fig. 2). Therefore it is possible that they did not observe the activity of domain B. In the present study, the concentration of fragment B tested for channel activity appeared high. This may be partly due to the fact that in our assay system using patch pipettes, the effective areas are much smaller than those in black membranes. The characteristics, including dose dependency and influence of lipid composition (10), of the channel formed by fragment B are now being studied in our laboratory in comparison with those of fragment A-B and tetanus toxin in this assay system. The detailed results of the comparative study will be published elsewhere.

The toxic action of tetanus toxin is thought to involve at least the following steps: initial binding, uptake and retrograde axonal transport to the target cell membranes, binding to target cells, internalization into target cells, and finally interaction with target molecules resulting in inhibition of the process(es) of neurotransmitter release (20, 31). The channel-forming property of fragment B demonstrated in this study may be relevant to the mechanism by which an active toxin fragment, probably the fragment A portion of the toxin, is internalized into the cytoplasm of target cells. The molecular (or enzymatic) mechanism by which the active fragment inhibits transmitter release remains to be investigated.

In the present study, fragment B was isolated in two forms (B_1 and B_2) which were not distinguishable by mol wt on SDS-gel electrophoresis but which were distinguishable by their electric charges. This may explain the microheterogeneity in structure of tetanus toxin molecules. To obtain further information on this difference in structure, we are now determining the NH_2- and COOH-terminal sequences of fragments B_1 and B_2.

With the isolation of fragment B in this study and of other fragments in previously studies (2–4, 14, 20, 24, 26), it is now possible to investigate complete sets of delineated fragments of ca. 50 and ca. 100 kilodaltons of any combinations of the three domains of the tetanus toxin molecule for investigating its structure-function relationship.

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