DISSOCIATION OF TETANUS NEUROTOXIN INTO TWO POLYPEPTIDE FRAGMENTS

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SUMMARY. Analyses of neurotoxin protein of Clostridium tetani by polyacrylamide gel electrophoresis showed that the toxin as purified from culture filtrates ("extracellular" toxin, molecular weight 160,000) could be dissociated into two polypeptide chains of molecular weight 53,000 (Fragment α) and 107,000 (Fragment β) by treatment with dithiothreitol and sodium dodecyl sulfate. The toxin as purified from bacterial extracts ("intracellular" toxin) was found to consist of a single 160,000 dalton polypeptide chain, which is undissociable by such treatment but, when pretreated with trypsin, becomes dissociable into two fragments apparently identical with α and β .

In recent years, tetanus toxin has been purified extensively and some of its physical and chemical properties including amino acid composition have been well characterized (1∿5). The toxin has been obtained in pure form as a highly neurotoxic simple protein of molecular weight about 150,000 daltons having six free SH-groups and two disulfide bridges (4, 5). However, very little has been known about the "subunit" structure of the toxin molecule, the information of which will provide a clue for the elucidation of structure-function relationship of the toxin. Thus we have been attempting to isolate biologically active fragments of the neurotoxin and during a course of the investigation we found that the toxin could be dissociated into two polypeptide chains. In this paper we report the dissociation of two types ("extracellular" and "intracellular") of tetanus toxin and discuss their molecular structure on the basis of the finding.

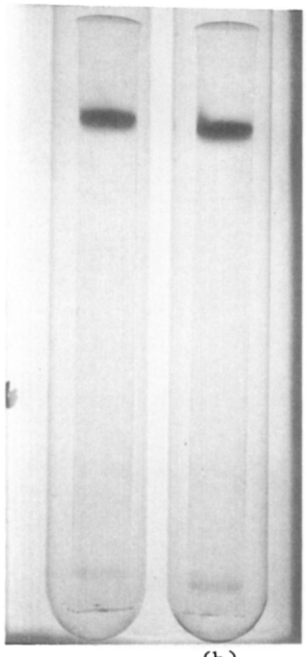
MATERIALS AND METHODS

Clostridium tetani strain Harvard A47 was employed for the production of tetanus toxin in modified Mueller medium (6). "Extracellular" toxin was prepared from culture filtrates of the organisms grown at 35 C for 4∿5 days. "Intracellular" toxin was obtained from bacterial extracts of the cells grown at 35 C for about 42 hours. The extracts were prepared at 0 C with agitation according to the method of Raynaud et al. (4). The toxin in the filtrates or extracts was concentrated and purified at 0∿4 C by subsequent steps comprising of ammonium sulfate fractionation (20∿40% saturation fraction), ultracentrifugation at 100,000 x g for 120 min to remove insoluble materials and gel filtra-

tion on Sephadex G-200 column in 0.1 M NaK phosphate buffer (pH 7.5). Purified toxin preparations have 360∿385 flocculating units(Lf)/Absorbance(280nm), 2∿4 x 10^4 minimum lethal doses(MLD)/Lf and Absorbance(280nm)/Absorbance(260nm) ratio of 2.0∿2.1. They each formed a single precipitation band against crude horse antitoxin in Ouchterlony's plates. Analytical polyacrylamide gel electrophoresis was performed using 7.5% gel by the methods originally described by Davis (7). Sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis was carried out employing 5% or 10% gel as described by Weber and Osborn (8), using human gamma-globulin, ovalbumin (Schwarz/Mann Co.), bovine serum albumin (Armour Pharmaceut. Co.), pepsin (2x crystallized) and trypsin (Worthington Biochem. Corp.) as standard molecular weight markers. Gels were stained using Coomassie brilliant blue and the relative intensities of protein bands were determined by scanning at 550 nm with Beckman spectrophotometer-Gilford multiple absorbance recorder equipped with a linear transport. Molecular weight was estimated from relative mobilities which were determined from scans of gels using distance from the top to the maximum peak height, employing standard markers as references. Dithiothreitol and soy bean trypsin inhibitor were obtained from Sigma Chem. Co., L-1-toluenesulfonylamido-2-phenylethyl chloromethyl ketone-treated trypsin from Worthington Biochem. Corp., Sephadex G-200 from Pharmacia Fine Chem. Co..

RESULTS AND DISCUSSIONS

Dissociation of "extracellular" toxin: Fig. 1a and Fig. 2a illustrate that the purified "extracellular" toxin runs as a single protein component on polyacrylamide gel in the absence (Fig. 1a) or presence (Fig. 2a) of SDS(0.1%). The molecular weight of the toxin was 160,000 ± 5,000 as determined from a plot of molecular weight versus relative mobility on SDS gel. This value is in close agreement with that (approximately 150,000) reported by others by different method (9). Reduction of the "extracellular" toxin (1 mg/ml in 50 mM Tris-HCl, 1 mM EDTA, pH 8.2) with dithiothreitol (100 mM) at 25 C for 60 min gave no influence on the electrophoretic behavior on polyacrylamide gel in the absence of SDS (Fig. 1b). However, when the thiol reduced toxin was subjected to gel electrophoresis in the presence of SDS, almost the entire preparation dissociated into two protein components (Fig. 2b, α and β), remaining a faint protein band (Fig. 2b, T) at the position corresponding that of the untreated toxin. The faster moving component was designated Fragment α, the slower one Fragment β, for convenience. The molecular weights of the two fragments and band T were: Fragment α = 53,000 ± 3,000, Fragment β = 107,000 ± 4,000 and T= 160,000 ± 5,000. Similar experiments were done using 10% gel with similar results. The ratio of the amount of Fragment β to that of Fragment α determined by densito-



(b)

Fig. 1. Polyacrylamide gel electrophoresis in the absence of SDS of "extracellular" toxin. (a) untreated and (b) reduced with dithiothreitol. Approximately 10 μg protein was applied on each gel. The electrode buffer was 0.01 M Tris, 0.054 M glycine (pH 8.3). Electrophoresis was at a constant current of 2.0 ma per gel at 4 C for 3.5 hours. Stained with Coomassie brilliant blue. Migration was from top to bottom.

metric analyses by amount of stain estimated from weighing the areas under the scan curves (β/α) = 2.0 ± 0.16. The observed ratio is in reasonable agreement with that predicted (2.02) for 1:1 stoichiometry, suggesting that the two fragments are complementary each other for the whole toxin molecule. The amount of T band varied from preparation to preparation and usually occupied about 5% of the preparation.

Dissociation of "intracellular" toxin: Fig. 3a shows that the purified "intracellular" toxin, just like "extracellular" toxin, migrates as a single protein component of molecular weight 160,000 on SDS gel. However, in contrast to the latter, the former remains to run as a single component of the same molecular weight even when reduced with dithiothreitol prior to SDS-gel electrophoresis (Fig. 3b). Since this difference in electrophoretic behavior between two types of toxin after reduction was considered to be due to the occurrence of at least one cleavage of peptide bond between the two particular cysteine residues forming disulfide bridge in "extracellular" toxin, effect of trypsinization on the electrophoretic behavior of "intracellular" toxin was

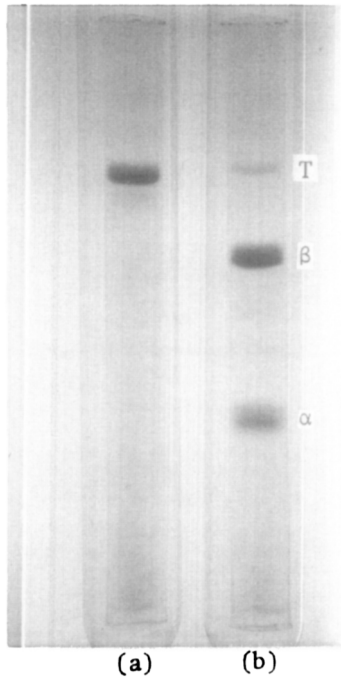


Fig. 2. SDS-polyacrylamide gel electrophoresis (5% gel) of "extracellular" toxin. (a) not reduced (10 μg sample) and (b) reduced with dithiothreitol (20 μg sample). Mercaptoethanol was omitted from the sample buffer (10) for the unreduced toxin. Electrophoresis was performed at a constant current 8 ma per gel for 5.5 hours. Migration was from top to bottom.

examined. Thus "intracellular" toxin (1 mg/ml in 50 mM Tris-HCl, 1 mM EDTA, pH 8.2) was preincubated with L-1-toluenesulfonylamido-2-phenylethyl chloromethyl ketone-treated trypsin (1 μg/ml) at 25 C for 60 min. At the end of the incubation soy bean trypsin inhibitor (1.5 μg/ml) was added and after several minutes the trypsinized toxin was reduced with dithiothreitol as described above and then subjected to electrophoresis in the presence of SDS. Fig. 3c illustrates that under the conditions the preparation dissociates completely into two components. The molecular weights of these two components were identical with those of Fragment α and β respectively and the two components were found to exist in equimolar ratio. Incidentally, as shown in Fig. 3d, about two-thirds fraction of the "intracellular" toxin preparation dissociated into two components of molecular weights identical with those of Fragment α and β by merely treating with trypsin and subsequent electrophoresis on SDS gel. This is in sharp contrast to the case of "extracellular" toxin, where no such change in the electrophoretic behavior was observable upon such treatment.

The data presented above indicate that tetanus toxin synthesized within the cells consists of a single polypeptide chain of molecular weight 160,000,

Fig. 3. SDS-polyacrylamide gel electrophoresis (5% gel) of "intracellular" toxin. (a) not trypsinized, not reduced (10 μg sample), (b) not trypsinized but reduced (10 μg sample), (c) trypsinized and reduced (20 μg sample) and (d) trypsinized, not reduced (20 μg sample). Other experimental conditions were the same as described in the legend for Fig. 2.

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while most of the toxin molecules released into the culture medium are each composed of two polypeptide chains (molecular weight 53,000 and 107,000, respectively) linked by at least one disulfide bridge. Thus "intracellular" and "extracellular" tetanus toxins appear to correspond "intact" and "nicked" forms of diphtheria toxin (10, 11, 12) respectively. However, the fact that tetanus toxin, being different from diphtheria toxin (13), requires addtional treatment with SDS for the dissociation into two polypeptide chains indicates further that these chains are held together firmly also by non-covalent bonds dissociable by SDS.

Artificial conversion of "intracellular" toxin by trypsinization to "nicked" form apparently identical with "extracellular" toxin suggests that conversion can occur naturally by the action of proteolytic enzyme(s) produced by tetanus bacilli. Incidentally, we observed that prolonged storage (at 4 C for more than half a year) of a purified "intracellular" toxin preparation resulted in "nick" formation. This is possibly due to the presence of such enzyme(s) contaminated in the preparation. Reduction was necessary for the dissociation of "extracellular" toxin. So the site(s) of cleavage of the peptide bond(s) involved in the

conversion must be between the two particular cysteine residues of "intracellular"toxin corresponding those which in "extracellular" toxin serve to link Fragment α and β by forming interchain disulfide bridge. About two-thirds fraction of the "intracellular" toxin preparation employed dissociated upon trypsinization even without reduction. This suggests that in majority of the "intracellular" toxin molecules, no disulfide bridge is formed between the particular pair(s) of cysteine residues involved in the dissociation. Recently Bizzini et al. have reported that as far as physical and chemical properties they examined are concerned, both types of tetanus toxin are identical (5). However, as presented above, they differ from each other in their fine molecular structure, reflecting the occurrence of definite structural alteration of the toxin during the course of its production.

Seki et al. reported definite increase in toxicity of "autolysates" of young tetanus bacilli by the action of trypsin (14). We have also observed reproducible enhancement of toxicity (more than three-fold increase) of "intracellular" toxin by trypsinization, suggesting the progenitor nature of the toxin. The trypsinization resulted also in "nick" formation (Fig. 3c). This suggests possible association of enhancement of toxicity with the structural alteration, as reported on type E progenitor toxin of Clostridium botulinum (15).

Elucidation of detailed biological, physical and chemical properties of Fragment α and β must await further studies employing purified fragments. Isolation of Fragment α and β is now in progress in our laboratory.

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