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STRUCTURE OF TETANUS TOXIN

N-Terminal Amino Acid Analysis of the Two Molecular Forms of Tetanus Toxin and its Composite Chains

Vladimir Neubauer and Torsten B. Helting

From the Research Laboratories, Behringwerke AG
3550 Marburg/Lahn, Germany

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Summary

N-Terminal amino acid analysis of the intracellular form of tetanus toxin revealed proline as the single terminal residue present in significant quantities. In agreement with new concepts on the structure of tetanus toxin, a second N-terminal amino acid (leucine) was exposed upon conversion to the extracellular form of the toxin molecule. These results were corroborated by analysis of the separate polypeptide chains of the extracellular toxin, and it is concluded that the light chain polypeptide constitutes the N-terminal region of the single chain toxin molecule originally synthesized by the bacterial cell. Treatment of the intracellular tetanus toxin with trypsin in vitro resulted in the exposure of amino acids in addition to those found after conversion to the extracellular form effected by the bacterial protease during fermentation.

Conflicting evidence is at hand concerning the amino terminal structure of tetanus toxin. Whereas some investigators (1, 2) have failed to identify the presence of any N-terminal amino acid, others have implicated aspartic acid (3), leucine (4), or glycine (5) as the single terminal residue. Klimek et al. (6) reported the presence of four N-terminal amino acids (Asx, Gly, Lys, Glx) in the formaldehyde derivative of tetanus toxin.

Most of these data are not quite consistent with present concepts of the subunit structure of tetanus toxin. Accordingly, intracellular toxin, isolated from the bacteria at an early stage during fermentation, is composed of a single polypeptide

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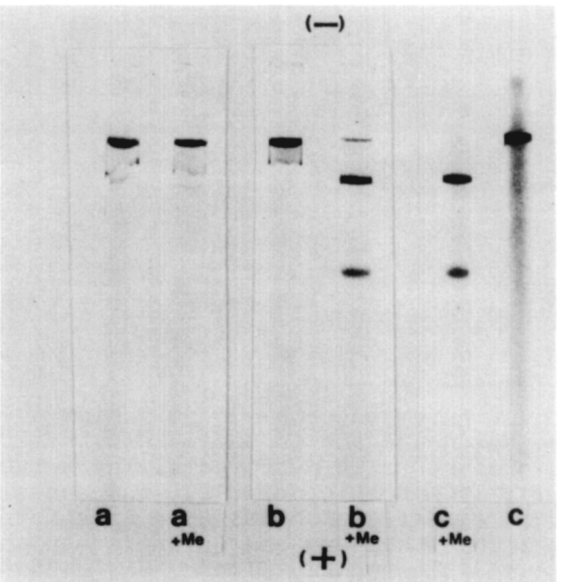

Fig. 1 Analysis on SDS gel electrophoresis of tetanus toxin (a) intracellular tetanus toxin; (b) extracellular tetanus toxin; (c) trypsinized intracellular tetanus toxin.
Samples which were reduced with mercaptoethanol prior to electrophoresis are indicated by the symbol "+Me".

overnight with the detergent. The extracts were lyophilized and used for N-terminal amino acid analysis as described below.

Determination of N-terminal amino acids. Dansylation of tetanus toxin in either molecular form was carried out in SDS buffer according to Gray (14) or Weiner et al. (15). For each determination, 2-10 nmoles of protein was used. Analysis of the hydrolysates was performed on polyamide sheets (15) or on silica gel plates (16). Edman degradation was achieved as outlined by Peterson et al. (17), or in the presence of SDS (15).

RESULTS AND DISCUSSION

Dansylation of intracellular tetanus toxin revealed DNS-Pro as the only identifiable amino acid derivative (Fig. 2a). In addition, a second dansyl derivative which did not co-chromatograph with any standard dansyl amino acid was present in a position close to, but distinct from DNS-Ileu (Fig. 2a). Isolation of this derivative by preparative thin layer chromatography followed by hydrolysis in 6 M HCl at 100°C for 4 hours produced

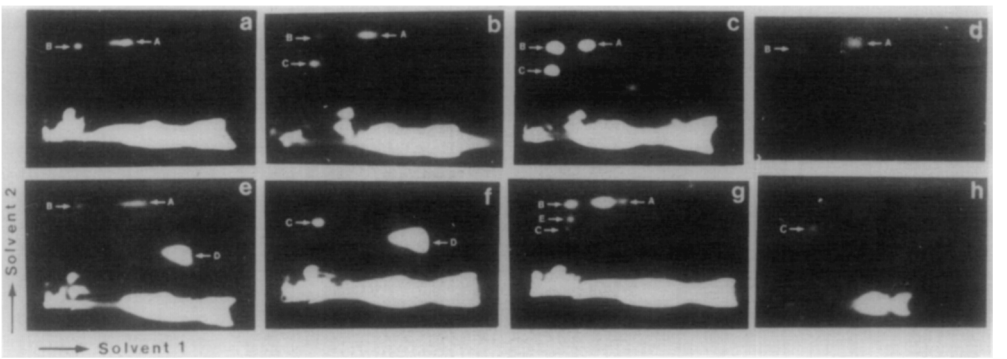

Fig. 2 Thin layer chromatography on polyamide sheets of tetanus toxin and the composite polypeptide chains after dansylation and acid hydrolysis. The solvent systems were: 1), 1.5% formic acid; 2), toluene-glacial acetic acid, 9:1 (v/v). The sheets were irradiated with ultraviolet light at 254 nm and photographed through a Kodak Wratten 44 filter using Ilford Pan F 135 film. The following spots were identified on each chromatogram by co-chromatography: A, DNS-Pro; B, DNS-Pro-Ileu; C, DNS-Leu; D, Artefact prominent only in preparations obtained from SDS polyacrylamide gels; E, DNS-Ileu.
The photographs show a) intracellular tetanus toxin; b) extracellular tetanus toxin; c) co-chromatography of b) with standard derivatives; d) isolated dipeptide presumed to be DNS-Pro-Ileu after acid hydrolysis; e) light chain polypeptide; f) heavy chain polypeptide; g) trypsinized intracellular tetanus toxin; and h)intracellular tetanus toxin after one cycle in the Edman degradation.

DNS-Pro as the sole fluorescent compound (Fig. 2d). Therefore, it was assumed that the unknown derivative presumably represented a dansylated dipeptide with N-terminal proline which had partially resisted cleavage during hydrolysis. Dansylation of Edman degraded intracellular toxin provided the clue to the structure of the dipeptide. Edman degradation confirmed proline as the terminal residue of the protein (Fig. 3a). Removal of proline exposed isoleucine as the second amino acid (Fig. 2h) in the sequence. Preparation of the dansylated dipeptide, DNS-Pro-Ileu resulted in a derivative with chromatographic properties indistinguishable from those of the unknown compound produced upon acid hydrolysis

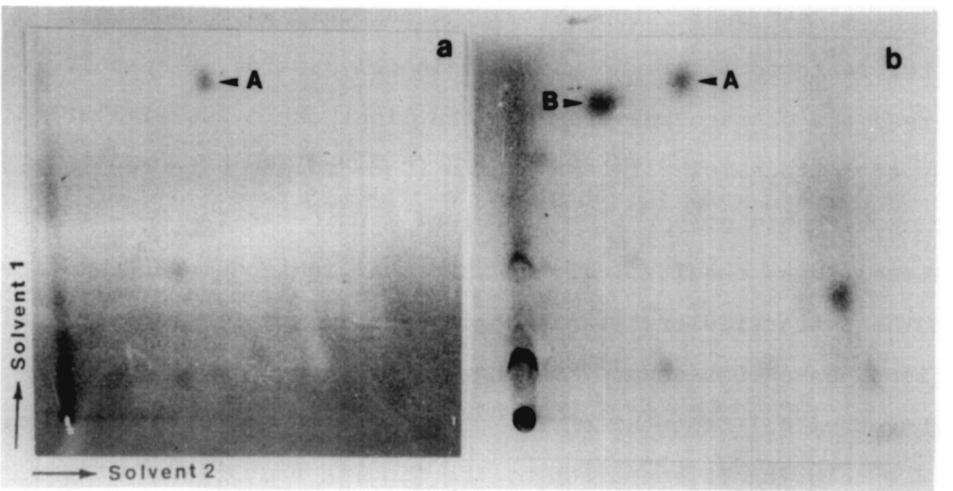

Fig.3 Thin layer chromatography on polyamide sheets of tetanus toxin after Edman degradation. The solvent systems were: 1) toluene-n-pentane-acetic acid, 60:30:16 (v/v); 25% acetic acid. Solvent 1) also contained 2,5-bis(2(5-tert-butylbenzoxazolyl))-thiophen (BBOT), 125 mg/l. The photographs were prepared as described in the legend to Fig. 2. The spots identifiable as PTH-amino acids by co-chromatography were: A, PTH-Pro; and B, PTH-Leu/Ileu. The photographs show a) intracellular tetanus toxin; and b) extracellular tetanus toxin.

of the dansylated intracellular toxin. Furthermore, acid hydrolysis of the dansylated standard dipeptide resulted in the partial release of DNS-Pro, indicating the relative stability of the Pro-Ileu bond towards acid hydrolysis.

Dansylation of extracellular tetanus toxin also revealed the presence of DNS-Pro and DNS-Pro-Ileu, respectively (Fig. 2b). In addition, a third derivative was observed which co-chromatographed with DNS-Leu. These results were corroborated by analysis of PTH-amino acids following Edman degradation of the extracellular toxin molecule (Fig 3b). Thus, the data are compatible with the idea that conversion of tetanus toxin to the extracellular form occurs by proteolytic cleavage of the polypeptide chain, hence exposing a second N-terminal region.

Figs. 2e-f depict the chromatograms obtained on analysis of the isolated light and heavy chain polypeptide, respectively. Whereas the pattern derived from the light chain was similar to that of intracellular tetanus toxin (DNS-Pro and DNS-Pro-Ileu), the heavy chain polypeptide contained leucine as the N-terminal residue. These results indicate that the light chain polypeptide and the intracellular tetanus toxin exhibit identical N-terminal regions. As a consequence, the heavy chain would represent the carboxyterminal fragment of the original tetanus toxin molecule. This conclusion is supported by the finding that leucine seems to constitute the N-terminal amino acid of the heavy chain and that this amino acid also becomes exposed upon conversion of the toxin to the extracellular form (see above).

Fig. 2g shows the derivatives obtained after dansylation and hydrolysis of trypsinized intracellular tetanus toxin. Interestingly, the pattern of the dansyl derivatives was not quite identical to that derived from the extracellular toxin, although both products were indistinguishable by SDS gel electrophoretic analysis (Fig. 1). In addition to DNS-Pro and DNS-Pro-Ileu already present prior to trypsination, the digestion with the enzyme resulted in exposure of only limited quantities of leucine. The principal amino acid released by trypsin was isoleucine, and traces of other amino acids (glycine, alanine) were also seen on the chromatograms. It is suggested that the clostridial protease may be more specific in performing the hydrolytic cleavage of the intracellular toxin polypeptide. Trypsin appears to introduce a microheterogeneity into the N-terminal region of the heavy chain. As expected, such a heterogeneity will pass undetected upon SDS gel electrophoresis of the trypsinized protein.

The results presented in this communication are in complete agreement with the recent hypothesis concerning the polypeptide chain structure of tetanus toxin (2,7,8) and argue against the model advocated by Bizzini et al. (18). However, the present work is at variance with most of the data from other groups (1-6) on the N-terminal amino acid(s) identified. A partial explanation for the contradictory reports that have appeared so far may possibly be sought in the use of toxin preparations of variable purities. The findings reported here are based on the analysis of highly purified preparations of both forms of tetanus toxin as well as of its composite chains. The experiments have been repeated with several batches of independent toxin preparations and the results have been consistent. Although occasionally, traces of other amino acids were seen on chromatograms, such contaminants must be attributed to minor, non-specific cleavage under the reaction conditions employed. The data collected thus seem to present a coherent picture of the N-terminal structure of tetanus toxin and indicate the order of arrangement of the composite polypeptide chains.

Acknowledgment

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chain (1, 2), whereas extracellular toxin, purified from the culture supernatant of autolysed cultures, consists of two subunit chains, linked to each other by way of a disulfide bond (2, 7, 8). The conversion of the intracellular toxin to a molecular species resembling the extracellular form has been documented *in vitro* using trypsin (7). In the bacterial culture itself, a clostridial protease has been demonstrated which transforms the intracellular toxin to its extracellular form (9).

The present effort was undertaken to identify the N-terminal amino acid(s) of tetanus toxin and to correlate the results with the subunit chain structure of the extracellular form of this molecule.

MATERIALS AND METHODS

Chemicals. Standard amino acids and their PTH- or dansyl derivatives were obtained from Sigma, St. Louis, Mo. USA. Phenyl isothiocyanate and dansyl chloride as well as solvents (Sequanal(R) grade) for N-terminal amino acid analysis were products of Pierce Eurochemie, Rotterdam, Holland. Polyamide sheets (F1700) were obtained from Schleicher & Schuell, Dassel, Germany, and TPCK-trypsin from Worthington. Benzamidine was supplied by Sigma. Ultrogel(R) was a product of LKB, Bromma Sweden.

Preparation of tetanus toxin. The extracellular form of tetanus toxin was isolated from culture filtrates as described (10). Upon reduction with mercaptoethanol, the toxin dissociated into the light and the heavy chain polypeptide (Fig. 1). To prepare the intracellular form of the toxin, fermentation was interrupted at 40 hours, the cells were harvested by centrifugation, washed in 0.15 M NaCl and resuspended in 1/10 of the original volume in a buffer containing 1 M NaCl in 0.1 M sodium citrate (11, 12). The buffer was supplemented with benzamidine (2mg/ml) to minimize any proteolytic activity present. The extracted crude toxin was separated from cells and debris after incubation at 4°C for 5 days and purified to apparent homogeneity by ion exchange chromatography on DEAE-cellulose and gel chromatography on Ultrogel(R) AcA 44, essentially analogous to the procedure used to prepare the extracellular toxin (Fig. 1). After preliminary treatment with trypsin, however, the two forms of tetanus toxin were indistinguishable on SDS gel electrophoresis (cf. ref. 7).

Isolation of the light chain and heavy chain polypeptide. Extracellular tetanus toxin was heated in the presence of 1% SDS and 5% mercaptoethanol and subjected to electrophoresis in buffer containing SDS according to Weber and Osborn (13). The position of the heavy chain and the light chain of tetanus toxin was established by staining a few gels. The relevant regions of the unstained gels were homogenized with 0.03% SDS and extracted