Conformational and Molecular Weight Studies of Tetanus Toxin and Its Major Peptides*

John P. Robinson§, Leslie A. Holladay¶, John H. Hash‡, and David Puett¶**

From the §Department of Microbiology and the ¶Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232

Two forms of tetanus toxin have been purified from Clostridium tetani cultures. These forms, obtained by filtrate and cellular extracts, were characterized by analytical ultracentrifugation using both conventional and meniscus-depletion sedimentation equilibrium. The molecular weight of filtrate toxin was found to be 128,000 ± 3,000, while the extract toxin, which tended to self-associate, appeared somewhat larger, 140,000 ± 5,000. The heavy and light chains were prepared from filtrate toxin, and their molecular weights were estimated to be 87,000 and 48,000, respectively, using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

The circular dichroic spectra of the extract and filtrate toxins are quite similar between 290-300 nm indicating that no major conformational difference exists between the two. The toxins contain both α-helicity and β-structure. Interestingly, the isolated chains contain appreciable helicity (e.g., the sum of the chain helicities is over 80% of that found in filtrate toxin), but they appear to have relatively low contents of β-structure. The sum of the spectra of the chains in both the near- and far-ultraviolet does not yield that found for filtrate toxin, although the similarity is far more striking than the difference. The prominent 293.5 nm negative circular dichroic band of tetanus toxin can be assigned to tryptophanyl residues almost exclusively in the heavy chain. The similarity in the magnitude of this band in the separated chain and toxin suggests that the microenvironments of the contributing tryptophans change very little when toxin is dissociated into its constituent chains.

Within the last decade tetanus toxin has been extensively examined and, although much has been learned about its biological, physical, and chemical properties, an examination of the current state of knowledge (1-12) quickly reveals that much remains to be done for elucidation of the structure and mechanism of action. The literature on tetanus toxin was recently reviewed (13, 14) and need not be detailed here.

Instead, we will only refer to those areas which directly apply to our results.

The toxin is prepared either by extraction from log phase cells (extract or intracellular toxin) or by purification from culture filtrate of older cultures (filtrate or extracellular toxin). It is generally agreed that the former is a single polypeptide chain while the latter has been cleaved by endogenous proteases and consists of two major peptides connected by disulfide linkage (1-9). One peptide in the cleaved form is much larger than the other and the two are often referred to as heavy and light chains based on the size difference. We have recently shown that cleavage of tetanus toxin by endogenous proteases can occur near to and on either side of the disulfide bond linking the heavy and light chains (15). Filtrate toxin preparations therefore can contain more than one form of toxin. In one form the light and heavy chains are covalently connected by disulfide linkage, but in the other they are noncovalently held.

We have prepared highly purified extract and filtrate toxins and examined the ultraviolet circular dichroic spectra of each. The purified filtrate toxin, which is the form consisting of noncovalently connected heavy and light chains, was separated into its heavy and light chain components and these too were examined.

There has been considerable disagreement about the molecular weight of tetanus toxin. The older literature reveals values that are at wide variance with the more acceptable current structural models. Most recent reports using gel filtration, ultracentrifugation, or the analytical ultracentrifuge give values which vary between 140,000 and 160,000 (1, 3, 5, 10). It is generally known that there is considerable aggregation and fragmentation in those samples studied. Consequently, in addition to the CD spectral analyses, we also report here a reexamination of the molecular weight of both filtrate and extract tetanus toxin.

MATERIALS AND METHODS

Chemicals—All chemicals used in this work were reagent grade unless otherwise stated. Acrylamide and N,N'-methylenebisacrylamide were purchased from Eastman. The acrylamide was recrystallized from chloroform. Guanidine hydrochloride was Heico's extreme purity product. Guanathione was purchased from Sigma and thiglycolic acid from Pierce. The apparatus used for preparative gel electrophoresis was a Buchler Poly Prep 200 preparative gel electrophoresis unit. Phenylmethanesulfonyl fluoride was purchased from Sigma. Azocoll was obtained from Calbiochem-Behring.

Toxin Production and Purification—C. tetani, Massachusetts strain, was grown in 20- to 40-liter quantities on the medium described by Latham et al. (16). Cultures to produce filtrate toxin and extract toxin were prepared as reported by others (5, 7). Both forms of the toxin were initially purified by a combination of gel filtration and ion exchange chromatography as described earlier (10).

Further purification was carried out by preparative gel electrophoresis. Electrophoresis was conducted at 45 mA using an elution buffer...
flow rate of 12 to 18 ml/h. The buffers consisted of the following: upper electrode buffer (6.32 g of Tris and 3.94 g of glycine/1000 ml), lower electrode buffer (48.4 g of Tris and 200 ml of 1 N HCl/1000 ml), and elution buffer (12.1 g of Tris and 50 ml of 1 N HCl/1000 ml). The resolving gel consisted of 4% polyacrylamide in 60- to 90-ml volume. The stacking gel contained 2.5% polyacrylamide and 4% urea in 32-

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Molecular Weight Determinations - Filtrate toxin, with H² and L chains being held covalently by disulfide linkage, was prepared by preparative gel electrophoresis, without reduction, as described under “Materials and Methods.” The purified toxin was immediately dialyzed into 0.2 M sodium phosphate buffer and examined in the analytical ultracentrifuge. Meniscus depletion gave a molecular weight of 128,000, and a plot of In C versus r² was linear over a 60-fold concentration range. When the same material was examined by low speed equilibrium, data analysis gave M∞ cell as 127,000 and M∞ cell as 130,000. An M∞ plot of In [(dc/dr)/r] versus r² according to Lamm (21) is shown in Fig. 1 and exemplifies a monodisperse preparation. The value of M∞ from this plot is 131,000 and agrees well with M∞ obtained from the meniscus-depletion experiment. We therefore conclude that fresh preparations of the species of filtrate toxin, in which the H and L chains are covalently held gave M∞ = 128,000 ± 3,000.

If these preparations are allowed to stand for 1 week at 4 °C they no longer show such homogeneity but show considerable fragmentation and aggregation. The reason for this change is at present uncertain but may involve residual activity of an endogenous protease(s). Extract toxin was prepared by preparative gel electrophoresis in the presence of reducing agents as described under “Materials and Methods.” Following dialysis into buffer containing 1 mM thioglycolic acid, the sample was immediately subjected to analytical ultracentrifugation. From two separate meniscus-depletion runs the molecular weight was determined as 138,000 and 143,000 and both gave linear plots of In C versus r² over a 60-fold concentration range. From a low speed sedimentation equilibrium run M∞ was calculated using the initial portion of a plot of In [(dc/dr)/r] versus r² (Fig. 2) to be 140,000, although clearly the solution is not monodisperse.

1 S. J. DiMari, M. A. Cumming, J. J. Hash, and J. P. Robinson, unpublished observations.
The data for the two forms of toxin are from analytical ultracentrifugation (low speed sedimentation equilibrium and meniscus depletion), and the results for the constituent chains of filtrate toxin are estimates from sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
are indicative of both α-helicity and β-structure. The sum of the H and L chain spectra (with corrections made for the molecular weight differences) is shown in Fig. 5C, and the inset shows the CD difference spectrum of filtrate toxin minus the sum of the consistent chain spectra. The major band in the difference spectrum occurs at 217.5 nm, and this suggests that β-structure is preferentially lost when the H and L chains are formed from filtrate toxin. The smaller band at about 225 nm may reflect a small loss in α-helicity when the chains are formed from toxin, and there may also be a contribution from aromatics and β-turns in this region (26-28).

Using highly accurate CD data for proteins of known crystallographic structure (28), we attempted to estimate the amounts of α-helicity, β-structure, and β-turns in the toxins and chains. With no constraints imposed on the fraction of residues in either helical, β-structure, β-turns, or aperiodic forms, the helix fraction was estimated to be between 0.23 and 0.24 in the two forms of toxin. This is in agreement with our earlier studies (10-12) using a somewhat different reference set of spectra, although in the present case a poor sum test was obtained. By constraining the sum of helical fraction, β-structure fraction, and remainder (i.e., aperiodic plus β-turns) to unity, the toxins and the H and L chains had respective apparent helical contents of 35 ± 1% (i.e., the helicity of both forms of toxin were within 1% of each other), 30%, and 24%. The respective β-contents were 30 ± 1%, <1%, and 2%. It is of interest that the sum of α-helicity in the H and L chains, corrected for their respective molecular weights, yields a combined helicity which is over 80% of that found in filtrate toxin.

**DISCUSSION**

These experiments reveal that the molecular weights of both nicked filtrate and unnicked extract toxins are somewhat less than the 150,000 which have been reported (2, 10). The higher values in earlier reports are most likely the result of aggregation in the samples which was occasionally acknowledged (10, 14). Also, the use of mixtures of filtrate toxins containing both covalently and noncovalently joined heavy and light chains could lead to overestimates of the molecular weight. Moreover, it is apparent that such studies must be completed soon after purification since there is significant fragmentation and aggregation within 1 week of storage at 4°C. Efforts to examine the two major peptides in the analytical ultracentrifuge have been unsuccessful due to fragmentation and aggregation, and the fragmentation was accelerated when the determinations were attempted in 6 M guanidine hydrochloride. The reason for this sample deterioration is unknown but one possible explanation may be residual protease activity which is accelerated when the peptides are unfolded in the presence of the denaturant. We emphasize, however, that using sodium dodecyl sulfate-polyacrylamide gel electrophoresis the estimates for the molecular weight of the two chains agree well with that expected for intact toxin.

The CD results are particularly illuminating and indicate that any conformational differences between the two forms of toxin must be subtle. The near-UV CD spectra of toxin and chains strongly suggest that the major tryptophanyl band in toxin can be attributed almost exclusively to the H chain. This is in excellent agreement with amino acid composition studies. Also, our finding that the sum of the spectra of the chains differs from that of toxin indicates that the microenvironments of at least some of the aromatic groups are not the same. This could arise from conformational alterations accompanying chain separation.

The far-UV CD data suggest that much of the α-helicity in toxin can be ascribed to the H chain. Earlier work with another toxin fragment, Fragment II which seems equivalent to Fragment C of Helting and Zwisler (7), suggested a predominance of β-structure (11), although the origin of the structure (i.e., intramolecular or intermolecular) was not defined. It was concluded earlier that Fragment C was located in the NH2-
terminal portion of the heavy chain (7). However, subsequent immunological (29) and NH2-terminal analyses (30) of these fragments now indicate that Fragment C, and consequently our Fragment II, are located in the COOH-terminal portion of the H chain. Therefore, a-helical structure in the H chain may be confined mostly to the NH2-terminal portion. Lastly, we wish to emphasize that the separated H and L chains appear to retain much of the helical structure that exists in toxin suggesting the possible occurrence of quasi-independent structural domains that may interact via \( \beta \)-structure since this seems to be preferentially lost in the isolated chains.

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