Structure and Action of Heteronemertine Polypeptide Toxins

DISULFIDE BONDS OF CEREBRATULUS LACTEUS TOXIN B-IV*

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The positions of the disulfide bonds in Cerebratulus lacteus toxin B-IV were investigated by hydrolysis of the unreduced protein with a variety of proteases. The resulting peptides were purified by gel filtration, ion exchange chromatography, and preparative paper electrophoresis and chromatography. Determination of the amino acid compositions of the cystine-containing peptides purified demonstrated the existence of disulfide bonds linking half-cystine residues 12 and 24, 21 and 51, and 35 and 38. The fourth bond, involving half-cystines 10 and 47, was assigned by difference.

The amino acid sequence of toxin B-IV, a crustacean-selective, axonal neurotoxin purified from the heteronemertine worm Cerebratulus lacteus, has recently been established (1). The purpose of the experiments reported herein was to determine the positions of the disulfide bonds of toxin B-IV and to possibly gain some preliminary insight into the role of these bridges in the biological activity of the protein. Experimental procedures and details of the methods employed for purification of individual peptides are given in the miniprint section.1

RESULTS

Toxin B-IV, the amino acid sequence of which is given in Fig. 1, was initially succinylated and hydrolyzed with a mixture of trypsin, chymotrypsin, and thermolysin. The positions for two of the four disulfide bridges could be determined by amino acid analysis of the resulting peptides, the purification of which is detailed in the miniprint section. To obtain the positions of the remaining bridges, a second sample of the native toxin was maleylated and treated as described in the miniprint section. Peptides containing three of the four toxin disulfide bonds were purified and characterized in at least one of the hydrolysates described.

Identification of Disulfide Pairs—The sequences of the isolated cystine-containing peptides are shown in Fig. 2. In all cases, the residues of half-cystine contributing to a disulfide pair could be identified from the amino acid composition of the isolated peptide (Tables I and II). However, in the case of Peptides (T-1,C-1)-Th2C and -Th3B (Table II) a single cycle of the Edman degradation was run to confirm that alanine was indeed NH₂-terminal. Analysis of the <PTC> derivatives liberated, after hydrolysis to the free amino acid (10), yielded only alanine. A second aliquot was analyzed by thin layer chromatography, after conversion of the anilinothiazolinone to the <PTC> derivative. These samples contained Ala<PTC> and an unidentified substance which failed to move off the origin in either heptane:1-butanol:formic acid (50:30:7, by volume) or in xylene:2-propanol (7:2, by volume).

DISCUSSION

One of the more difficult problems encountered in the course of this work was the extreme resistance of native toxin B-IV to the commercially available proteases. Pretreatment of the toxin with 8 M urea, 6 M guanidine HCl, by boiling, or by acidification to pH 2.3, failed to relieve this intractibility. Hence, after an initial reluctance to expose the native toxin to neutral or slightly alkaline pH, succinylation and maleylation were employed; either procedure served to render the protein susceptible to trypsin. No evidence for disulfide exchange was observed during this study in that no cystinyl peptides having compositions incompatible with our disulfide assignments were purified.

The procedure used for maleylation of the protein is worthy of comment. Our intent was not quantitative modification of cysteine, but to facilitate the disulfide exchange with maleylated protein.

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†Figs. 3 through 6 are presented in a miniprint format immediately following this paper. This section describes the isolation of peptides used to obtain the disulfide pairs. For the convenience of those who prefer to obtain this data in the form of 9 pages of full-size photocopies, they are available as JBC Document No. 76111-1608. Orders should specify the title, authors, and reference to this paper, the JBC Document Number, and the number of copies desired. Orders should be addressed to the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance of the order of the Journal in the amount of $1.35 per set of photocopies.

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been firmly established herein, the existence of a Cys\textsubscript{10}-Cys\textsubscript{47} disulfide bond linking Cys-10 and Cys-47 is assigned by difference. For these reasons, a smaller than normal excess of maleic anhydride was utilized, the reaction was carried out for 5 min at pH 8.0, rather than 9 to 10, and tryptic hydrolysis ensued immediately, without prior removal of free maleic acid. Additional exposure of the maleylated protein to trypsin was observed.

The application of trypsin, chymotrypsin, thermolysin, and elastase, either singly or in groups, allowed isolation and characterization of peptides containing three of the four disulfide bonds known to exist in toxin B-IV. Thus, the evidence for the bond linking Cys-10 and Cys-47 is indirect. In view of the known participation of all 8 residues of half-cystine in the protein in disulfide bonds (2), the lack of evidence for disulfide exchange, and since the remaining three disulfide pairs have been firmly established herein, the existence of a Cys\textsubscript{10}-Cys\textsubscript{47} disulfide is at least highly probable. While it would have been more desirable to have direct confirmation of this bond, we felt that the large amount of toxin which might be required for confirmation could be more usefully employed for chemical modification studies.

This paper describes the first investigation of the disulfide bonds of heteronemertine toxins. It may therefore be worth noting that the disulfide bond pattern established is different from that observed with scorpion (11) or snake neurotoxins.

**TABLE I**

| Amino acid     | SMP-1 | SMP-3D | SMP3-B3 |
|----------------|-------|--------|---------|
| Lysine         | 4.0 (4) | 2.0 (2) | 1.0 (1) |
| Histidine      | 0.3 (0) | 1.0 (1) | 0.9 (1) |
| Arginine       | 1.0 (1) | 0.4 (0) | 0.2 (0) |
| Cysteic acid   | 3.5 (4) | 1.7 (2) | 2.2 (2) |
| Aspartic acid  | 3.1 (3) | 0.8 (1) | 1.3 (1) |
| Glutamic acid  | 2.0 (0) | 0.4 (0) | 0.2 (0) |
| Glycine        | 2.0 (2) | 0.8 (1) | 1.3 (1) |
| Alanine        | 2.1 (2) | 0.8 (1) | 1.3 (1) |
| Isoleucine\textsuperscript{a} | 0.7 (1) | 1.0 (1) | 0.2 (0) |
| Leucine        | 1.0 (1) | 0.2 (0) | 0.2 (0) |
| Tyrosine       | 1.0 (1) | 0.2 (0) | 0.2 (0) |
| % yield\textsuperscript{c} | 64 | 26 | 16 |
| Mobility\textsuperscript{d} Before oxidation | 0.39 | 0.45 | 0.68 |
| After oxidation | 0.11 | 0.15 | 0.43 |
| % yield\textsuperscript{c} | 64 | 26 | 16 |

\textsuperscript{a} In this and the following table, the numbers in parentheses indicate the numbers of residues found in the sequence.

\textsuperscript{b} Values for isoleucine are uncorrected for incomplete release after 22 h of acid hydrolysis in this and the following table.

\textsuperscript{c} Yields of peptides are uncorrected for losses during the course of purification in this and the following table.

\textsuperscript{d} Mobilities given in this and the following table were determined by high voltage paper electrophoresis at pH 2.0, and are expressed relative to lysine = 1.00.

**TABLE II**

| Amino acid | T-1, C-1, Th2C | T-1, C-1, Th3B | T-1, C-1, Th4C | T-1, C-1, Th4C-3 |
|------------|---------------|---------------|---------------|----------------|
| Lysine     | 1.3 (1)       | 1.0 (1)       | 2.0 (2)       | 0.8 (1)       |
| Histidine  | 1.8 (2)       | 2.1 (2)       | 1.9 (2)       | 2.0 (2)       |
| Cysteic acid | 1.9 (2) | 1.1 (1) | 0.3 (0) | 0.9 (1) |
| Aspartic acid | 0.1 (0) | 0.1 (0) | 0.1 (0) | 0.1 (0) |
| Threonine  | 0.0 (0)       | 0.1 (0)       | 0.1 (0)       | 0.1 (0)       |
| Serine     | 0.1 (0)       | 0.1 (0)       | 0.1 (0)       | 0.1 (0)       |
| Glutamic acid | 1.0 (1) | 1.0 (1) | 1.2 (1) | 1.2 (1) |
| Glycine    | 1.3 (1)       | 1.4 (1)       | 1.0 (1)       | 1.2 (1)       |
| Alanine    | 0.9 (1)       | 0.8 (1)       | 1.0 (1)       | 1.0 (1)       |
| Isoleucine | 0.1 (0)       | 1.6 (2)       | 1.0 (1)       | 1.0 (1)       |
| Leucine    | 0.1 (0)       | 1.0 (1)       | 1.0 (1)       | 1.0 (1)       |
| % yield    | 37            | 14            | 10            | 10            |
| Mobility   | 0.67          | 0.78          | 0.71          | 0.68          |
| Chromatographic mobility | 0.18 | 0.70 | 0.89 | 0.35 |
| Cysteine Residues | 12 + 24 | 12 + 24 | 35 + 38 | 21 + 51 |

\textsuperscript{a} Values for isoleucine are uncorrected for incomplete release after 22 h of acid hydrolysis in this and the following table.

\textsuperscript{b} Yields of peptides are uncorrected for losses during the course of purification in this and the following table.

\textsuperscript{c} Mobilities given in this and the following table were determined by high voltage paper electrophoresis at pH 2.0, and are expressed relative to lysine = 1.00.

\textsuperscript{d} Mobilities given in this and the following table were determined by high voltage paper electrophoresis at pH 2.0, and are expressed relative to lysine = 1.00.

The amino acid sequences of purified, cystine-containing peptides, based on the compositions shown in Tables I and II. In each case, a single peptide is shown to prove the existence of the bridge, although variants of both SMP-3D and T-1,C-1, Th2C were also characterized.

Fig. 2. Amino acid sequences of purified, cystine-containing peptides, based on the compositions shown in Tables I and II. In each case, a single peptide is shown to prove the existence of the bridge, although variants of both SMP-3D and T-1,C-1, Th2C were also characterized.

The amino acids of these two groups render the protein sensitive to brief treatment with trypsin. This limited hydrolysis should then serve to denature the protein, leaving all of the lysine and arginine peptide bonds sensitive to trypsin, after demaleylation. For these reasons, a smaller than normal excess of maleic anhydride was utilized, the reaction was carried out for 5 min at pH 8.0, rather than 9 to 10, and tryptic hydrolysis ensued immediately, without prior removal of free maleic acid. Additionally, exposure of the maleylated protein to trypsin was brief (40 min) and carried out at pH 7.0. Our hopes were realized, in that the bulk of trypsin-sensitive bonds were apparently cleaved, and no evidence for disulfide exchange was observed.
(12). As noted elsewhere (1) primary structures of scorpion, snake, and Cerebratulus toxins display no homology.

One further interesting point is raised by our findings. The disulfide bond linking Cys-35 and Cys-38 must result in the formation of a hairpin turn in the polypeptide chain (residues 35 to 38) which can be predicted by the method of Chou and Fasman (13). It would be of interest to ascertain whether the Cys 35-Cys 38 pair can be preferentially reduced and to determine the effects of such partial reduction upon toxicity, particularly since current theory implicates a carboxylate group in the function of the sodium channel (14, 15), and the loop formed as a consequence of the hairpin should be highly cationic (Fig. 1). Experiments studying the effects of partial reductions are currently in progress.

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2 K. M. Blumenthal and W. R. Kem, unpublished data.
SUBJECT: ROD OF PHOTORECEPTOR HTTP://www.jbc.org/2020/2020/2020/EXPANORAL PROCEEDURES

Experimental Procedure:

Dissociation of Rods — The dissociation procedure was performed on freshly excised rods in a modified HEPES buffer (pH 7.3) at the room temperature.
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K M Blumenthal and W R Kem

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