Identification and Partial Characterization of a Low Affinity Metal-binding Site in the Light Chain of Tetanus Toxin*

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Tetanus toxin was shown to contain a metal-binding site for zinc and copper. Equilibrium dialysis binding experiments using $^{65}$Zn indicated an association constant of 9–15 μM, with one zinc-binding site/toxin molecule. The zinc-binding site was localized to the toxin light chain as determined by binding of $^{65}$Zn to the light chain but not to the heavy chain after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to Immobilon membranes. Copper was an efficient inhibitor of $^{65}$Zn binding to tetanus toxin and caused two peptide bond cleavages in the toxin light chain in the presence of ascorbate. These metal-catalyzed oxidative cleavages were inhibited by the presence of zinc. Partial characterization of metal-catalyzed oxidative modifications of a peptide based on a putative metal-binding site (HELIX) in the toxin light chain was used to map the metal-binding site in the protein.

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The abbreviations used are: MOPS, 4-morpholineethanesulfonic acid; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FAB-MS, fast atom bombardment mass spectrometry.

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branes were finally incubated with $^{65}$Zn.

A second method in which the native conformation of the protein is maintained was also used to evaluate $^{65}$Zn binding and for competition studies: tetanus toxin (1 mg/ml) was equilibrated in 2 mM MOPS, 150 mM NaCl, pH 7.0, using 2-ml Sephadex G-50 minicolumns equilibrated with the same buffer. To 20 $\mu$l of the protein solution was added 2 $\mu$l of MOPS, NaCl buffer containing approximately 0.2 $\mu$Ci of $^{65}$ZnCl$_2$ corresponding to a final zinc concentration of 2 $\mu$M. After incubation for 1 h at 20 °C, the protein solution was applied to a TSK G3000 SW analytical column (300 x 7.5 mm) (Waters), and the eluate was monitored for $^{65}$Zn radioactivity and for protein (280 nm). Competition for $^{65}$Zn binding to the toxin by various divalent cations was performed using a 500-fold molar excess of these cations.

The quantitative parameters of $^{65}$Zn binding were investigated by using also two different methods: first, by equilibrium dialysis essentially as previously described for $^{40}$Ca binding to CT (Thielen et al. 1990). Tetanus toxin in 2 mM MOPS, 150 mM NaCl, pH 7.0, was at a final concentration of 10 $\mu$M. Incubation lasted 42 h at 4 °C. The binding data were plotted according to Scatchard (1949).

The second method was by centrifugation-filtration, as described by Penefsky (1977). Incubations of toxin with various concentrations of ZnCl$_2$, containing trace $^{65}$Zn were carried out for 1 h at 20 °C before passage of the mixture through miniature Sephadex G-50 columns. The binding data were plotted according to Scatchard (1949).

Copper-mediated cleavage of tetanus toxin was as follows: 100 $\mu$l of 10 $\mu$M toxin in 2 mM MOPS, 150 mM NaCl, pH 7.0, was made 10 $\mu$M CuCl$_2$. Ascorbate was added to a final concentration of 100–200 $\mu$M. The mixture was incubated for various time periods, at 37 °C, and subsequently fragments were analyzed by 12.5% SDS-PAGE under reducing conditions. SDS-PAGE lanes were transferred to ProBlott membranes and after Coomassie Blue staining, the protein bands were excised and subjected to NH$_2$-terminal microsequencing as described above. Zinc and various peptides were tested for their ability to inhibit copper-mediated cleavage of tetanus toxin light chain. The modification of TT225–243 by copper and ascorbate was investigated by incubation of a solution of 100 $\mu$M peptide in the MOPS/NaCl buffer with 100 $\mu$M copper and ascorbate ranging in concentration from 100 $\mu$M to 1 mM for various time periods at 20 °C. The oxidized products were analyzed and purified using a HPLC C18 reverse-phase analytical column (Nova Pak (150 x 3.9 mm) (Waters)) employing a gradient from 5 to 80% acetonitrile in 0.1% trifluoroacetic acid. Controls were performed in which TT225–243 was treated with CuCl$_2$ alone or with ascorbate alone.

RESULTS

Zinc Binding Parameters—The binding of $^{65}$Zn to the light chain of tetanus toxin separated by SDS-PAGE and subsequently transferred to an Immobilon membrane is shown in Fig. 1. Under reducing conditions the light chain but not the heavy chain of the toxin shows $^{65}$Zn binding. The monoclonal form of the toxin was also able to bind $^{65}$Zn under the same conditions.

When purified toxin was preincubated with a solution of $^{65}$Zn and subsequently chromatographed by gel filtration under physiological pH and salt concentration, the protein peak monitored at 280 nm coeluted with a peak of radioactivity, indicating binding of $^{65}$Zn to the toxin molecule. An equivalent amount of $^{65}$Zn alone in buffer solution did not pass through the column, suggesting that free zinc bound to the column matrix under these conditions. A similar approach has been described to investigate binding of $^{64}$Cu by dopamine $\beta$-monooxygenase (Skotland and Flatmark, 1983). Accumulated free zinc could be removed from the column by flushing with 10 mM EDTA overnight. The binding of $^{65}$Zn by toxin measured by this technique was sensitive to pH, being maximal at 7.0 and dropping off sharply below 6.5 (data not shown).

Using the same gel filtration assay, the ability of various divalent cations to displace or compete with $^{65}$Zn was measured. Fig. 2 indicates that copper was more effective than nonradioactive zinc in its ability to prevent $^{65}$Zn binding to tetanus toxin. Nickel, and to a lesser extent cobalt, were able to weakly reduce $^{65}$Zn binding to the toxin molecule.

Equilibrium dialysis was used to determine the parameters of $^{65}$Zn binding to tetanus toxin. Fig. 3 shows the combined results of two independent experiments. The dissociation constant was calculated to be 15 $\mu$M and the number of sites was 0.81 molecule $^{65}$Zn/molecule of tetanus toxin. Equilibrium dialysis was performed at 4 °C due to a tendency of the toxin molecule to stick to the dialysis membranes when incubated at 20 or 37 °C for extended periods. The results were supported by an independent binding assay performed at ambient temperature in which toxin-bound $^{65}$Zn and unbound $^{65}$Zn were separated using small (200 $\mu$l) gel filtration columns (Penefsky, 1977). The results of these experiments (not shown) indicate a $K_d$ of 9 $\mu$M and 0.86 $^{65}$Zn-binding sites/toxin molecule.

Targeted Metal-catalyzed Oxidative Modification—When TT was incubated with copper and dithiothreitol or ascorbate
Metal Binding by Tetanus Toxin

**Fig. 3.** Scatchard analysis of zinc binding to tetanus toxin. Binding of $^{65}$ZnCl$_2$ (from 5 to 110 $\mu$M) to tetanus toxin (10 $\mu$M) was measured by equilibrium dialysis. Data are plotted according to Scatchard (1949), and the straight line is derived by linear regression analysis.

**Fig. 4.** Effect of zinc on the metal-catalyzed oxidation of tetanus toxin. Tetanus toxin (10 $\mu$M) was incubated for 6 h as described under “Materials and Methods,” at 37 °C in 2 mM MOPS, 150 mM NaCl, pH 7.0, alone (control, lane 1), with 10 $\mu$M CuCl$_2$ and 200 $\mu$M sodium ascorbate (lane 2) or with 1 mM ZnCl$_2$, 10 $\mu$M CuCl$_2$, and 200 $\mu$M sodium ascorbate (lane 3). Lane 4 corresponds to molecular weight standards. After incubation, samples were reduced and analyzed by SDS-PAGE in a 12.5% SDS-polyacrylamide gel.

For 6 h at 37 °C, conditions which are known to cause metal-catalyzed site-specific oxidative modifications in proteins (Stadtman, 1990), a series of discrete bands were generated. Toxin alone (Fig. 4, lane 1) or TT in the absence of ascorbate or Cu (data not shown) was not cleaved, revealing that the purified toxin was free of extrinsic contaminating protease activity. Oxidative cleavage of the tetanus toxin, as shown by SDS-PAGE under reducing conditions (Fig. 4, lane 2), generated fragments of 47 (p 47), 28 (p 28), and 25 kDa (p 25), corresponding to a decrease of the light chain of about 10%, as estimated by densitometry of the gel colored by Coomassie Blue. NH$_2$-terminal microsequencing of fragments after transfer to ProBlott membranes indicated that p 47 and p 28 contained the NH$_2$-terminal sequence of the toxin light chain, while the NH$_2$ terminus of p 25 was blocked. These results are consistent with generation of two copper-mediated poly-

**Fig. 5.** Effect of native and oxidized TT225–243 peptides and of native TT233–247 peptide on the metal-catalyzed oxidative cleavage of tetanus toxin. Tetanus toxin (10 $\mu$M) was incubated for 6 h as described under “Materials and Methods,” at 37 °C in 2 mM MOPS, 150 mM NaCl, pH 7.0, alone (control, lane 1), with 10 $\mu$M CuCl$_2$ and 200 $\mu$M sodium ascorbate (lane 2), and with 10 $\mu$M CuCl$_2$ and 200 $\mu$M sodium ascorbate in the presence of 50 $\mu$M TT225–243 peptide (lane 3), 50 $\mu$M oxidized TT225–243 peptide (lane 4), or 50 $\mu$M TT233–248 peptide (lane 5). Lane 6 corresponds to molecular weight standards. After incubation, samples were reduced and analyzed by SDS-PAGE in a 12.5% SDS-polyacrylamide gel.
Peptide TT225-243 was treated with copper and ascorbate under conditions which led to polypeptide cleavage in the intact toxin. Assessed by reverse-phase HPLC chromatography, no modification of the peptide was observed at concentrations of ascorbate (50–500 μM) equivalent to those which induced toxin light chain cleavage. However, concentrations of ascorbate approximately 100-fold higher (5–50 mM) caused a time-dependent modification of the peptide, giving rise to two products TT225–243A and TT225–243B (Fig. 6A). Mass spectrometry analysis indicated molecular masses of 2142.2 for unmodified TT225–243, 2158.6 for TT225–243A, and 2158.6 for TT225–243B, suggesting the addition of a single atom of oxygen in each case (Fig. 6, B and C). NH₂-terminal microsequencing suggested modifications of amino acids His-232, Glu-233, and His-236 in TT225–243A and His-232 and Glu-233 in TT225–243B, as shown by the absence of these residues.

**DISCUSSION**

The binding of metal ions to proteins has been established as important in both structural and functional aspects of protein chemistry. Zinc (reviewed by Vallee and Auld, 1990) as well as copper (Scott et al., 1988; Calabrese et al., 1989) have been shown to be essential components of the active sites of a variety of enzymes. Our demonstration of the binding of one zinc atom/toxin light chain suggests that these metals bind to a HELIX motif located within this chain, a site analogous to that established as forming part of the zinc receptor.
covalent interaction site in thermolysin and a number of other zinc enzymes (Jongeneel et al., 1989). This motif as the site of metal binding in tetanus toxin is supported experimentally by binding of $^{65}$Zn to TT225–243, a synthetic peptide based on the toxin sequence and containing this motif.

The pH dependence of zinc binding to the toxin and the low affinity of this binding are suggestive of binding to histidinyl residues, consistent with a loss of zinc binding upon protonation of the histidine imidazole nitrogens at values of pH below 6.5.

Interestingly, we have found that, in the qualitative zinc binding assay involving coelution of $^{65}$Zn with protein by gel filtration, the toxin light chain bound several times more $^{65}$Zn than a comparable molar quantity of intact toxin. This can be due to the rather drastic conditions used for the preparation of the TT light chains. However, it cannot be excluded that in whole tetanus toxin, the heavy chain down-regulates the binding of zinc to the light chain.

Our demonstration of zinc and copper binding by the light chain of tetanus toxin suggests that these metals may play a structural role in the toxin light chain as discussed above.

In the context of our studies of antigen processing and presentation using tetanus toxin as a model antigen, the ability of the toxin to bind metals with ensuing alterations of the toxin conformation is an important aspect for consideration.

According to Stille et al. (1987) and Rothbard and Taylor (1988), the amino acid sequence 233–248 in the tetanus toxin light chain is a potential T lymphocyte epitope. The amino acid sequence containing the HELIH motif appears to fulfill the requirements of a T-cell epitope as proposed in the amphipathic helix hypothesis (Cornette et al., 1989) and of a region of high hydrophathy contrast which typifies metal-binding sites in proteins (Yamashita et al., 1990). Thus, binding of a metal atom may be important for the conformation of this putative T-cell epitope. More generally, the presence of metal bound to tetanus toxin may play a role during the processing of the toxin by antigen-presenting cells and play on the B or T antigenic reactivity.

Preliminary data on the interaction of peptide 233–248 with antibodies raised in rabbit show that zinc favors the peptide–antibody interactions.

Experimentally, the copper/zinc competition for binding to TT and the ability of copper to cleave about 10% of the light chain of tetanus toxin in the presence of ascorbate to give discrete fragments suggested to us that copper could be used as an oxidative probe of the metal-binding site in the toxin light chain. This is based upon the observation that metal-catalyzed oxidative modification of a protein occurs at or close to the metal-binding ligands of proteins (Marx and Chevion, 1985; Farber and Levine, 1986; Stadtman, 1990; Miller et al., 1990). Zinc competitively inhibits the copper-mediated oxidative cleavage of TT in agreement with previous experiments showing that zinc protects bacteria from copper-mediated oxidative damage (Korbashi et al., 1989). Comparably ion protection has been reported for Mg in iron-mediated oxidative cleavage of Escherichia coli glutamine synthetase (Jhon et al., 1991). The precision of metal-catalyzed oxidative modification of biomolecules has also been used to establish specific metal-binding sites in peptides (Uchida and Kawakishi, 1990) and DNA (reviewed by Sigman, 1990).

We were unable to fully characterize the Cu$^{2+}$-catalyzed cleavage of tetanus toxin light chain due to NH$_2$-terminal blocking of the p 25 fragment and the inability to isolate and characterize the 5 kDa light chain fragment. However, the apparent molecular weights of the polypeptides as well as the NH$_2$-terminal sequence data for p 28 and p 47 allowed us to estimate the locations of the two sites. The cleavage site responsible for the generation of p 25 and p 28 is estimated to be within the light chain sequence leucine 230-methionine 260, consistent with the localization of the zinc-binding site within the HELIH sequence, while the second site is within the sequence aspartic acid 405-isoleucine 435.

Mass spectrometric analysis of peptide TT225–243 oxidative products gives evidence for major modification at the level of His-232 and -236. This last finding is consistent with the observation that peptides which contain the entire HELIH sequence, and at a lesser level peptide containing only ELIH are able to inhibit the oxidative cleavage. From the above observation, it is quite evident that the oxidized peptide is far less efficient as an inhibitor of tetanus toxin oxidative cleavage. Thus, in the absence of simple Cu binding test, the above observations strongly suggest that the copper-binding site is similar to the zinc-binding site. As shown by Jongeneel et al. (1989), the homology of the HELIH motif of tetanus toxin light chain with the same motif found in established zinc metalloproteases such as thermolysin extends to regions flanking HELIH, supporting the hypothesis that zinc may play a role in a putative enzymatic active site in the tetanus toxin.

In preliminary experiments we have tested the ability of the protoxin and bichain toxin molecules and isolated light and heavy chains to cleave a limited number of established zinc metalloprotease substrates including endothelins (Vijayaraghavan et al., 1990), angiotensin, bradykinin, enkephalin, collagen, and gelatin. Although conclusive evidence for enzymatic activity has not been established, we have observed a weak proteolytic activity directed against collagen and gelatin. The problems of finding the appropriate conditions to enhance such activity and, more importantly, to eliminate the possibility of activity due to trace amounts of contaminating proteases remain to be resolved.

Although the molecular mechanism of tetanus toxin toxicity is largely unknown, the ability of minute amounts of the toxin to give rise to profound pathological consequences is consistent with the idea that tetanus toxin exerts its pathological effects by an enzymatic mechanism. Furthermore, the active component of the toxin molecule has been suggested to be the light chain which appears to act intracellularly, with the heavy chain serving to target the light chain to appropriate presynaptic neurons and enables the entry of the light chain into these cells (Mochida et al., 1989; Ahnert-Higler et al., 1989). The importance of histidine residues in the mechanism of toxicity of the closely related botulinum toxin, which shows a very high homology with tetanus toxin in the putative metal-binding HExxH region, has been suggested by loss of toxicity resulting from modification of histidine residues with diethyl pyrocarbonate (Dasgupta and Rasmussen, 1984). A similar loss of toxicity has been observed with DEPC-modified tetanus toxin.

The possibility of a proteolytic activity associated with the tetanus toxin molecule, as suggested by the binding of zinc or copper atoms to the toxin light chain, must also be considered in its processing as an antigen. Although in the experimental conditions used no autoproteolytic activity of TT was observed, it cannot be excluded that in specific intracellular compartments of antigen processing cells the modification of normal antigen processing pathways due to such enzymatic activity presents novel investigative approaches.
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