RESEARCH COMMUNICATION

Surface topography of histidine residues of tetanus toxin probed by immobilized-metal-ion affinity chromatography

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Tetanus toxin contains 14 histidine residues: six of them are localized in the light chain (L), one is present in the *N*-terminal half of the heavy chain (H_N) and the remaining seven histidines are localized in the *C*-terminal half of the heavy chain (H_C). Using immobilized-metal-ion affinity chromatography with Chelating Superose-Zn(II), we show that histidines of H_C are exposed to the protein surface and are responsible for the binding of tetanus toxin and of H_C to the immobilized metal. The histidines of the L chain are not available for co-ordination of matrix-bound Zn^{2+} ; however, two of them and three of the histidines of fragment H_C are accessible to diethyl pyrocarbonate. Chromatography on Superose-Zn(II) is also shown to be a simple and efficient method for the rapid isolation of tetanus toxin and of its H_C fragment, which can be extended to the botulinum neurotoxins.

INTRODUCTION

Tetanus toxin (TeTx) is a potent neurotoxin produced by toxigenic strains of *Clostridium tetani*. It binds to the presynaptic membrane at the neuromuscular junction, penetrates into the motoneuron and moves retroaxonally to reach the cytoplasm of the inhibitory interneurons of the spinal cord. Here it blocks the release of neurotransmitters, thus causing the spastic paralysis of tetanus (Simpson, 1989; Niemann, 1991).

TeTx is composed of a heavy chain (H, 100 kDa) and a light chain (L, 50 kDa) held together by a single interchain disulphide bridge and by non-covalent forces as shown in Fig. 1. Its primary structure has been determined (Eisel *et al.*, 1986; Fairweather & Lyness, 1986). TeTx has a 35% α -helix content and a 30% β -structure, with the interchain disulphide bond exposed at the protein surface (Robinson *et al.*, 1982; Schiavo *et al.*, 1990).

Crystals of TeTx suitable for X-ray crystallography have not yet been reported. From three-dimensional image reconstruction of negatively stained two-dimensional crystalline arrays of TeTx grown on phospholipid/ganglioside monolayers the toxin appears as a three-lobed structure (Robinson *et al.*, 1988).

To probe the localization of histidines and cysteines in TeTx and its fragments, we have used immobilized-metal-ion affinity chromatography (IMAC) (Porath *et al.*, 1975; Sulkowski, 1985; Hemdan *et al.*, 1989; Arnold, 1991). This technique is based on the co-ordination between electron-donor groups of the protein surface and chelated transition-metal ions bound to iminodiacetic acid residues attached on an agar-based matrix. Among the protein surface groupings able to interact with transition-metal ions in IMAC chromatography, histidine and cysteine have been shown to function as predominant ligands (Hemdan *et al.*, 1989; Arnold, 1991).

TeTx has 14 histidine residues, whose positions in the sequence are shown in Fig. 1. The first two, His-233 and His-237, are included in a segment strictly conserved among all the clostridial

neurotoxins whose sequence is available (Niemann, 1991). TeTx also contains 10 cysteine residues; four of them are disulphide-bridged (Kriegelstein *et al.*, 1990). The IMAC approach presented here has allowed us to obtain information on the localization (interior or surface) and surface density of the histidines of TeTx

Fig. 1. Schematic structure of tetanus toxin and its fragments L- H_N and H_C .

TeTx contains 14 histidine residues, whose positions are indicated: seven are in the L- H_N fragment and seven are in the H_C fragment.

Abbreviations used: TeTx, tetanus toxin; L, light chain of tetanus toxin; H, heavy chain of tetanus toxin; H_C , 50 kDa *C*-terminal fragment of the H chain; L- H_N , L chain plus the *N*-terminal half of the H chain; IMAC, immobilized-metal-ion affinity chromatography; DEPC, diethyl pyrocarbonate.

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and of its fragments, and to propose a simple and effective method of purification of TeTx and of the 50 kDa C-terminal fragment of the H chain (H_C) adaptable to micro as well as macro quantities and extendable to other toxins such as botulinum neurotoxins.

EXPERIMENTAL

Materials

Tetanus toxin and fragments. Tetanus toxin was purified as described by Schiavo *et al.* (1990) and stored at -80°C in 10 mM-NaHepes/50 mM-NaCl, pH 7.2. Fragments $L-H_N$ (L chain plus N-terminal half of the H chain) and H_C were obtained by digestion of tetanus toxin with papain (type IV, Sigma) (Weller *et al.*, 1989) and were isolated by size-exclusion liquid chromatography (HPSELC) using a TSK G-3000 SW (Pharmacia) column on a Beckman System Gold instrument provided with a diode-array UV-VIS 168 detector. Cytochrome *c* [horse heart (type VI, C 7752), tuna heart (type XI, C 2011)] and myoglobin [horse heart (M 1882)] and diethyl pyrocarbonate (DEPC) were from Sigma. Cytochrome *c* [yeast, *Candida krusei*] was purchased from Sankyo (Tokyo, Japan).

Methods

Chromatography. A Chelating Superose HR 10/2 column (Pharmacia), connected with a Heckman System Gold h.p.l.c. apparatus, was washed with 7.5 ml of 50 mM-EDTA/500 mM-NaCl, pH 6.0, and equilibrated with 10 ml of 50 mM-sodium acetate/200 mM-NaCl, pH 6.0. The column was charged with 7.5 ml of 0.1 M- ZnCl_2 and the unbound metal ion was washed out of the column with 10 ml of metal-free buffer (50 mM-sodium acetate/200 mM-NaCl, pH 6.0). Finally the column was equilibrated with 10 mM-NaHepes/150 mM-NaCl, pH 7.4 (buffer A). All protein samples (100 μg of tetanus toxin, L chain, its fragments $L-H_N$, H_C and DEPC-treated H_C , cytochromes *c* and myoglobin) were applied to the column in the equilibrating buffer and eluted at room temperature with a linear gradient of 0–25 mM-imidazole in buffer A at a flow rate of 0.5 ml/min.

Chemical modification. The reaction of H_C with DEPC was performed at a protein concentration of 0.3 mg/ml in 20 mM-sodium phosphate/100 mM-NaCl, pH 7.8, at 25°C by adding portions of a freshly prepared solution of 20 mM-DEPC in anhydrous ethanol; the final concentration of ethanol never exceeded 2% (v/v). The reaction was stopped by addition of imidazole at a final concentration of 5 mM. The time course of H_C histidine modification was followed by recording of the differential absorbance increase at 243 nm as previously described (Burstein *et al.*, 1974; Miles, 1977; Papini *et al.*, 1989). Modified residues were estimated from the differential absorption coefficient of $3200\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 243 nm for *N*-carbethoxyhistidine (Ovadi *et al.*, 1967) in a Perkin-Elmer Lambda 5 spectrophotometer. The DEPC-modified H_C fragment was dialysed against 20 mM-NaHepes/100 mM-NaCl, pH 7.0, before application to the Chelating Superose-Zn(II) column.

RESULTS AND DISCUSSION

Fig. 2 shows the chromatographic behaviour of TeTx, L chain, $L-H_N$ fragment and H_C on a Chelating Superose-Zn(II) h.p.l.c. column. When loaded at pH 7.4, only TeTx and H_C are retained, whereas the L chain and $L-H_N$ fragment are eluted with the void volume. The retention times of TeTx and of H_C are identical and intermediate between that of *Candida krusei* cytochrome *c*, which has two non-adjacent histidines, and that of horse myoglobin, which has two adjacent histidines on the same face of an α -helix. The coincidence of the retention times of TeTx and of its H_C

fragment indicates that histidines of the H_C fragment are responsible for TeTx binding. The folding of the polypeptide chain of H_C has to bring these histidines into close proximity in order to account for their efficient zinc co-ordination. This results also indicates that no histidines or cysteines are available on the surface of the L chain or of the $L-H_N$ fragment to interact with the immobilized Zn^{2+} ions. Presumably they are hidden in the protein interior or involved in interaction with other residues or ligands in such a way as to be unavailable for Zn^{2+} co-ordination.

TeTx and H_C can be eluted with a 0–25 mM linear gradient of imidazole. This indicates that the residues mainly involved in the interaction with chelated immobilized Zn^{2+} are histidines rather than cysteines. If this is indeed the case, chemical modification of the histidine residues of TeTx and of fragment H_C should inhibit their binding to the chelating Superose-Zn(II) column.

DEPC modifies six histidines of TeTx and three histidines of H_C . These DEPC-modified proteins are eluted with the void volume, thus showing that histidines are the only residues responsible for binding and that the cysteine residues of the toxin occupy an internal position in the molecule.

Fig. 3 shows the chromatographic behaviour of H_C modified

Fig. 2. Chromatograms of TeTx, $L-H_N$, L and H_C fragments after h.p.l.c. on Chelating Superose-Zn(II)

The samples were eluted with a gradient of 0–25 mM-imidazole in buffer A (see the Experimental section). L chain and $L-H_N$ fragment were eluted with the void volume, whereas TeTx and H_C were eluted with almost identical retention times, intermediate between those of *Candida krusei* cytochrome *c* (arrow a) and horse myoglobin (arrow b). Numbers refer to elution times (min) of peaks.

Fig. 3. Chromatograms of unmodified and DEPC-modified H_c fragments on Chelating Superose-Zn(II)

All samples (30 μ g of protein) were eluted with a gradient of 0–25 mM-imidazole in buffer A. Chromatogram (a) refers to the unmodified H_c fragment, and (b) and (c) refer to samples of H_c containing an average of one and two DEPC-modified histidines respectively. Numbers refer to elution times (min) of peaks.

with DEPC to different extents. Conversion on average of one histidine residue into *N*-carboxyhistidine per H_c molecule leads to the appearance of about one-half of the protein in the void volume, whereas the remainder is eluted at the same position as unmodified H_c . The modification of two histidines per protein molecule is sufficient to cause its elution with the void volume. DEPC does not react with thiol residues, and hence this result indicates that the two free cysteines (Cys-868 and Cys-1300) present in H_c are not exposed on the surface and available for binding to Superose-Zn(II).

Eight histidines, out of the 14 present in TeTx, are not modifiable by DEPC (results not shown), and hence are likely to occupy an internal position. Of the remaining six histidines which are accessible to DEPC, three are located in fragment L- H_N and are not exposed at the protein surface. On the contrary, two histidines located in fragment H_c are sufficiently exposed and adjacent to be able to co-ordinate a matrix-immobilized transition-metal ion.

The opposite chromatographic behaviour of the L chain and fragment L- H_N with respect to TeTx and fragment H_c makes this system ideal for their separation after proteolytic cleavage, particularly in comparison with the currently available procedures (Ozutsumi *et al.*, 1985; Weller *et al.*, 1989).

As shown in Fig. 4, the method is also useful for a rapid purification of TeTx from $(NH_4)_2SO_4$ precipitates of *Clostridium tetani* culture filtrates. The central portion of the peak contains over 97% TeTx as judged by densitometric scanning of SDS/PAGE gels stained with Coomassie Blue. A similar chromato-

Fig. 4. Chromatogram of $(NH_4)_2SO_4$ precipitate of *Clostridium tetani* culture filtrates on Chelating Superose-Zn(II)

A 2 mg portion of $(NH_4)_2SO_4$ precipitate of *Cl. tetani* supernatant was dialysed against buffer A and analysed by Chelating Superose-Zn(II) chromatography. Peaks a and b were collected, and 1.5 μ g of protein from each was applied to SDS/PAGE under reducing conditions (Schiavo *et al.*, 1990) and compared with 1.5 μ g of the starting material (lane AS). Peak a is pure L chain, and peak b is pure tetanus toxin. Numbers refer to elution times (min) of peaks.

graphic behaviour is found with botulinum neurotoxins serotypes A and E (results not shown), and hence this procedure can be extended also to these other clostridial neurotoxins. This method should also be very useful in the industrial isolation of H_c produced in *Escherichia coli*, which has been recently proposed as a possible anti-tetanus vaccine (Fairweather *et al.*, 1987).

In conclusion, the use of IMAC chromatography with a Superose-Zn(II) column has shown that two histidine residues of the 50 kDa C-terminal fragment of TeTx are exposed on the protein surface. These residues are in such a proximity as to be able to co-ordinate matrix-bound Zn^{2+} . Histidine co-ordination of immobilized ions is very useful for the rapid isolation of TeTx and its fragments and of botulinum neurotoxins for analytical as well as preparative purposes.

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