Metal substitution of tetanus neurotoxin

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Tetanus neurotoxin was depleted of its catalytic Zn^{2+} ion, and the apotoxin was reconstituted with different transition metal ions. The Mn^{2+}- and Co^{2+}-tetanus neurotoxins are highly active in the proteolysis of vesicle-associated membrane protein/synaptobrevin, the natural substrate of this toxin, whereas Cu^{2+} and Fe^{2+} minimally supported proteolytic activity. The visible absorbance spectrum of Co^{2+}-tetanus neurotoxin shows a maximum at 538 nm with a molar absorption coefficient of 82 $M^{-1} \cdot cm^{-1}$. These results indicate that the Zn^{2+} environment at the active site of tetanus neurotoxin is different from those of known Zn^{2+}-endopeptidases and provide a structural basis for the definition of tetanus neurotoxin, and the related clostridial neurotoxins, as an independent family of metalloproteases.

INTRODUCTION

Tetanus neurotoxin (TeNT) is a 150 kDa bacterial protein toxin produced by *Clostridium tetani* which causes the spastic paralysis of tetanus. TeNT chemically modified with formaldehyde (tetanus toxoid) is a very effective antitetanus vaccine, produced in dozens of millions of doses each year [1]. TeNT is the prototype of the clostridial neurotoxins which comprise seven botulinum neurotoxins responsible for the flaccid paralysis of botulism [2]. These neurotoxins are very similar and are composed of two polypeptide chains joined by a single interchain disulphide bond. The heavy chain (100 kDa) is responsible for the neurospecific binding and for the entry of the light chain (50 kDa) into the neuronal cytosol [2]. The light chains of the clostridial neurotoxins are proteases that are very specific for three protein components of the neuroexocytosis apparatus, the cleavage of which results in a sustained blockade of the release of neurotransmitters at the synapse [2]. They are Zn^{2+}-dependent endopeptidases containing an atom of zinc bound to the His-Glu-Xaa-Xaa-His-binding motif, characteristic of this class of proteases [2–4]. The zinc atom of TeNT is co-ordinated by the two histidines of the motif and by a glutamate-bound water molecule, which is essential for the proteolytic and neurotoxic activity of TeNT [4–8]. Recent mutagenesis and spectroscopic experiments indicate that Tyr-242 is a strong candidate for the fourth Zn^{2+} ligand [7,9]. The chemical characterization of the active site and of the residues involved in Zn^{2+} binding is very important for the molecular definition of the activity of this dangerous pathogen as well as for the design of novel and safer antitetanus vaccines based on active-site-mutated TeNT.

Information on the Zn^{2+} environment of Zn^{2+}-endopeptidases can be obtained by analysing the effects of replacing Zn^{2+} with other transition metal ions such as Co(II), Cu(II), Ni(II), Fe(II) and Mn(II) [10–16]. Co^{2+} in particular, is a sensitive probe of the metal environment because its derivatives exhibit useful spectroscopic properties [17,18]. Evolutionary relationships among known metallopeptidases have been recently studied by Rawlings and Barrett [4] by comparing primary and tertiary structures. They proposed a division into five groups, mainly based on the mode of metal co-ordination. The term clan was introduced to describe a group of families of metallopeptidases with a common ancestor. Thermolysin is the prototype of the MA clan, which comprises metallopeptidases binding a Zn^{2+} ion via the HEXXH motif and an additional glutamic residue. Astacin is a well-characterized protease of the MB clan, the members of which co-ordinate Zn^{2+} via the motif and an additional histidine. TeNT was placed in a third group comprising metallopeptidases, the additional metal ligand of which is as yet unidentified; on the basis of sequence comparisons, tetanus and botulinum neurotoxins are assigned to a separate family, termed M27. The Co^{2+}-thermolysin visible absorbance spectrum shows a maximum at 550 nm and a shoulder at around 500 nm [11], whereas the Co^{2+}-astacin spectrum has a maximum at 514 nm with a shoulder at 550 nm [14]. Thus the spectrum of the Co^{2+} derivatives of these metallopeptidases effectively reflects the different mode of Zn^{2+} co-ordination.

Here we describe the preparation of TeNT with the Zn atom substituted with Ni, Co, Cu, Mn and Fe. The pattern of enzymic activities of these metal-substituted TeNT preparations and the spectrum of Co^{2+}-TeNT provide clear evidence that the metal-binding site of TeNT is different from that of known metalloproteases. These results further characterize the active site of TeNT and support the definition of the clostridial neurotoxins as a separate family of metallopeptidases.

EXPERIMENTAL

Preparation of proteins

TeNT was isolated from culture filtrates of *Clostridium tetani* [19], frozen in liquid nitrogen and stored at −80 °C in 10 mM Hepes (sodium salt)/50 mM NaCl, pH 7.2. Rat vesicle-associated membrane protein (VAMP)-2 fragment 1–96 was expressed in *Escherichia coli* as a glutathione S-methyltransferase fusion protein (GST-VAMP-2/1–96) by inserting the gene construct corresponding to segment 1–96 of rat VAMP-2 into the *Sma*I and *Eco*RI sites of plasmid pGEX-KG [20] and transforming it into the AB1899 strain of *E. coli*. The GST-fused protein was

Abbreviations used: GST, glutathione S-methyltransferase; OP, 1,10-phenanthroline; TeNT, tetanus neurotoxin; X^{2+}-TeNT, cation-substituted tetanus neurotoxin; VAMP, vesicle-associated membrane protein.

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purified by affinity chromatography on GSH–agarose matrix (Sigma, Milan, Italy).

Determination of metal content

Experiments were performed in glassware or plasticware treated with 30 % nitric acid and washed thoroughly with Milli-Q-grade water (Millipore) (conductance $> 10\ M\Omega$), treated with Amberlite MB-3 (Sigma). Metal-free dialysis tubing (Spectrum) was prepared by extensive washing with metal-free water at 80 °C [21]. Buffers were prepared using chemicals of the highest purity available with respect to the presence of heavy metals in water obtained as described above. Before determination of metal content, the protein samples were extensively dialysed at 4 °C against PBS. Samples were analysed for Zn^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} and Ni^{2+} with a Perkin–Elmer 4000 atomic absorption flame spectrophotometer with impact bed loading. Metal contents were measured after standardization in the linear range of concentration for each ion (0–0.5 p.p.m. for Zn^{2+}, Ni^{2+}, Co^{2+}; 0–1 p.p.m. for Cu^{2+}; 0–5 p.p.m. for Fe^{2+}, Mn^{2+}).

Zn^{2+} removal and metal replacement

TeNT (21.3 mg/ml in PBS) was dialysed overnight at 4 °C against a 200-fold volume of 2 mM 1,10-phenanthroline (OP) (Sigma) in PBS followed by three dialysis steps (4 h) against PBS without OP. Incubation with more concentrated OP solution or at higher temperatures causes loss of toxin, which precipitates in the dialysis tubing. Metal-substituted TeNT was obtained by overnight dialysis against equimolar solutions of each metal (142 μM) in PBS at 4 °C, followed by three dialysis steps (2 h) against metal-free PBS.

Spectroscopy

Visible absorbance spectra were determined with a Perkin–Elmer Lambda 5 spectrophotometer on 600 μl samples of 100 μM apo-TeNT in PBS to which increasing amounts of 20 mM $CoSO_4$ were added up to a Co^{2+}/toxin ratio of 3:1.

Proteolytic activity of TeNT

Recombinant GST–VAMP-2/1–96 (1 mg/ml in PBS) was incubated for 1 h at 37 °C with X^{2+}-TeNT (substrate to enzyme ratio of 10:1), previously reduced as described [22], and the extent of proteolysis was estimated as previously described [23].

RESULTS AND DISCUSSION

Various procedures have been described for the replacement of active-site Zn^{2+} of Zn^{2+}-endopeptidases with other transition metal ions [10,18]. Here, we have followed the protocol first established for thermolysin [11], which consists of the preparation of the apoprotein, free of metal chelator, followed by incubation with the appropriate metal ion. Dialysis in the presence of OP is very effective in removing Zn^{2+} from TeNT. The apo-TeNT is reconstituted with different transition metal ions by dialysis of the apo-TeNT with an equimolar solution of the appropriate metal ion followed by extensive dialysis with a metal-free buffer solution. Different metal ions associate with TeNT with different final stoichiometries (Table 1). Zn^{2+} re-associates with high efficiency, followed by the other metal ions in the following order: $Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{2+}$. About twice as much Cu^{2+} as Zn^{2+} associates with apo-TeNT. This result indicates that Cu^{2+} may bind at both the proteolytic active site of TeNT and other sites, consistent with the finding of Wright and colleagues [24] that TeNT contains an additional low-affinity binding site for Cu^{2+}. This order of efficiency of metal ion binding to apo-TeNT is different from that found with thermolysin ($Zn^{2+} > Co^{2+} > Mn^{2+} > Fe^{2+} > Ni^{2+}$ [10]) and astacin ($Zn^{2+} > Fe^{2+} > Co^{2+} > Ni^{2+} > Cu^{2+} > Mn^{2+}$ [14,16]).

TeNT and the other clostridial neurotoxins exhibit the unusual property of cleaving only very large segments of their substrates and are completely ineffective on short segments spanning the known cleavage site [5,22,25–32]. This is due to the involvement in neurotoxin binding of segments of the substrate protein distal to the cleavage site [29,31,32]. For this reason, to measure the proteolytic activity of TeNT, we have used here the entire cytosolic portion of rat VAMP-2. Segment 1–96 was expressed as a fusion protein with GST and purified in a metal-free form. Table 2 reports the level of proteolytic activity obtained with the various metal-substituted TeNTs, expressed as percentage with respect to that of Zn^{2+}-TeNT, taken as 100 %, and corrected for the relative amount of metal bound to the toxin. The highest activity is exhibited by Mn^{2+}-TeNT, which is significantly more active than Zn^{2+}-TeNT itself. Co^{2+} has been substituted frequently for Zn^{2+} in metalloenzymes and, in nearly all instances, it fully supports catalytic activity, although its affinity is generally lower than that of Zn^{2+} [10,18]. Also in the case of TeNT, Co^{2+} is able to functionally replace Zn^{2+} at the active site and Co^{2+}-TeNT is as active as native TeNT, whereas Cu^{2+}- and Fe^{2+}-TeNT have low activities. Co^{2+} added to apo-TeNT, obtained by incubation with the chelating agent dipicolinic acid, was only poorly able to support the toxin-induced blockade of exocytosis in chromaffin cells [33]. This difference in activity of the Co^{2+} derivative of TeNT in the two assays can be attributed to the different conditions and type of assay used. In the experiment reported by Höhne-Zell et al. [33], Co^{2+} was added to TeNT in the presence of the metal ion chelator, which may interfere with the process of ion binding to the protein [10]. Moreover, permeabilized chromaffin cells may still contain several cations, including Zn^{2+}, and are likely to possess several metal-ion-

Table 1 Binding of transition metal ions to Zn^{2+}-depleted TeNT

Zn^{2+} was removed from TeNT by dialysis in the presence of OP and the residual Zn^{2+} content was measured (column labelled OP). The neurotoxin was reconstituted with different metal ions by further dialysis in the presence of the appropriate ion in equimolar amount with respect to the toxin multiplied by 100. Values are means ± S.D. for three different preparations.

Binding (mol/mol of toxin)						
Cu^{2+}	Zn^{2+}	Ni^{2+}	Co^{2+}	Mn^{2+}	Fe^{2+}	OP
150 ± 5	84.7 ± 5	75.5 ± 5	48.1 ± 5	35.2 ± 5	22 ± 5	7 ± 5

Table 2 Activity of metal-substituted TeNTs

The proteolytic activities of the various metal-substituted TeNT preparations, obtained as described in Table 1, was estimated as a percentage of substrate cleaved under the same conditions by apo-TeNT reconstituted with Zn^{2+}-TeNT, taken as 100 %, after correction for residual Zn^{2+}-TeNT and for residual apo-TeNT. Values are means ± S.D. obtained with three different preparations run in duplicate.

Relative activity (%)					
Zn^{2+}	Mn^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Fe^{2+}
100 ± 5	120 ± 10	100 ± 10	61 ± 10	26 ± 9	7 ± 5

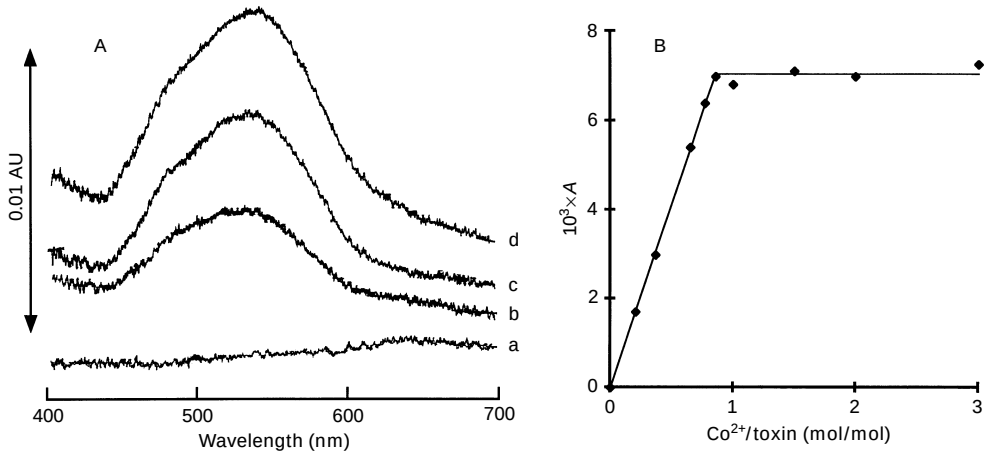

Figure 1 Visible absorbance spectra of Co^{2+}-TeNT

(**A**) Spectra were recorded with a 600 μl sample of 100 μM apo-TeNT in metal-free PBS (a) or plus $CoSO_4$ to reach a final Co^{2+}/TeNT ratio of 0.37 (b), 0.91 (c) and 1 (d). AU, arbitrary unit. (**B**) Increase in absorbance of apo-TeNT on incremental addition of $CoSO_4$. Absorbance increases up to a molar ratio of Co^{2+}/TeNT of about 0.85. Thus the effective concentration of Co^{2+}-TeNT used for the calculation of the molar absorption coefficient is 85 μM.

binding species, such as inositol phosphates, that may partially interfere with ion binding to TeNT.

The order of capacity of metal ions to support the proteolytic activity of TeNT is thus: $Mn^{2+} > Zn^{2+} = Co^{2+} > Ni^{2+} > Cu^{2+} > Fe^{2+}$. This differs from those of thermolysin ($Co^{2+} > Zn^{2+} > Fe^{2+} > Mn^{2+} > Ni^{2+}$ [11]) and astacin ($Co^{2+} > Zn^{2+} > Cu^{2+} > Hg^{2+} > Ni^{2+}$ [14]). The chemical basis of such differences is not known, but it is probably related to different amino acid side chains involved in metal binding at the active site and/or different geometry of liganding in the three different proteases. However, these results provide further evidence that the environment of the metal ion at the active site of TeNT differs from those of thermolysin and astacin.

Further strong support for this contention is provided by the analysis of the visible absorption spectrum of Co^{2+}-TeNT, shown in Figure 1(A). Apo-TeNT was titrated with increasing amounts of Co^{2+} and the visible spectrum was recorded after each addition. Absorbance increased during titration until a metal/toxin ratio of 0.85:1 was obtained and no further absorbance increments were recorded with successive additions of Co^{2+} (Figure 1B). A limited inactivation of the toxin caused by Zn^{2+} removal and/or the prolonged incubation with OP may be responsible for the lack of attainment of a 1:1 ratio. The absorbance spectrum of Co^{2+}-TeNT shows a maximum at 538 nm and a shoulder at 480 nm. The molar absorption coefficient of Co^{2+}-TeNT at 538 nm is estimated to be 82 $M^{-1} \cdot cm^{-1}$, a value intermediate between those of the thermolysin and astacin Co^{2+} derivatives [11,14]. The shape of the spectrum and absorbance maximum of Co^{2+}-TeNT are not dissimilar from those of Co^{2+}-thermolysin (maximum at 550 nm and shoulder at approx. 500 nm) [11], but clearly differ from those of Co^{2+}-astacin (maximum at 514 nm and shoulder at 550 nm) [14].

The results presented here show that TeNT, the prototype of the clostridial neurotoxin metalloproteases, differs from thermolysin and astacin in several ways. (1) TeNT shows a different order of preference for bivalent transition metal ions; (2) metal-substituted TeNT exhibits a different order of proteolytic activity with VAMP-2 from the corresponding thermolysin and astacin derivatives; (3) the visible absorbance spectrum of Co^{2+}-TeNT is unique in terms of shape and wavelength of maximum absorbance.

Recently, on the basis of the mutagenesis studies of Yamasaki et al. [7] and a comparison of the X-ray absorption properties of TeNT, thermolysin and astacin [9], we proposed that TeNT coordinates Zn^{2+} in a mode not previously found for metalloproteinases with the involvement of two histidines [5,7], the water molecule bound to the glutamate of the motif [6,7] and the conserved Tyr-242 [2,9]. The data presented here are consistent with such a proposal and provide further structural evidence that TeNT, and the related clostridial neurotoxins, form a separate family of metalloproteases [4].

We thank Dr. G. A. Smith for help during the preparation of the paper and F. Cattalini, Department of Biology of the University of Padova for atomic absorption measurements. This study was supported by Telethon-Italia with grant 763 and by CNR.

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Received 7 August 1996/28 October 1996; accepted 15 October 1996