Structure-based sequence alignment for the β-trefoil subdomain of the clostridial neurotoxin family provides residue level information about the putative ganglioside binding site

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Received 21 June 2000; revised 9 August 2000; accepted 9 August 2000

Edited by Matti Saraste

Abstract Clostridial neurotoxins embrace a family of extremely potent toxins comprised of tetanus toxin (TeNT) and seven different serotypes of botulinum toxin (BoNT/A–G). The β-trefoil subdomain of the C-terminal part of the heavy chain (H_C), responsible for ganglioside binding, is the most divergent region in clostridial neurotoxins with sequence identity as low as 15%. We re-examined the alignment between family sequences within this subdomain, since in this region all alignments published to date show obvious inconsistencies with the β-trefoil fold. The final alignment was obtained by considering the general constraints imposed by this fold, and homology modeling studies based on the TeNT structure. Recently solved structures of BoNT/A confirm the validity of this structure-based approach. Taking into account biochemical data and crystal structures of TeNT and BoNT/A, we also re-examined the location of the putative ganglioside binding site and, using the new alignment, characterized this site in other BoNT serotypes.

Key words: Tetanus neurotoxin; Botulinum neurotoxin; β-trefoil; Sequence alignment; Homology modeling; Ganglioside binding site

1. Introduction

Clostridial neurotoxins, the most potent toxins known, are a family of homologous proteins produced by anaerobic bacteria of the genus *Clostridium* [1,2]. This family comprises tetanus neurotoxin (TeNT), the casual agent of the pathological condition known as tetanus, made by *Clostridium tetani*, and botulinum neurotoxins (BoNTs), produced as seven immunologically distinct serotypes (BoNT/A–G) by *Clostridium botulinum* and other species of the same genus, responsible for the neuroparalytic condition, botulism [3,4]. The process of cell intoxication is achieved via a four-step mechanism [5,6]: (1) binding to specific neuronal receptors, (2) internalization into endosome-like vesicular organelles through receptor-mediated endocytosis, (3) membrane translocation which requires acidification of the vesicle lumen, and (4) modification of the cytosolic target. The main difference between TeNT and the BoNTs in this process is that, after binding at the presynaptical neuronal membrane, TeNT undergoes retrograde axonal transport to act in the central nervous system [7].

Clostridial neurotoxins are synthesized as single polypeptide chains (~150 kDa), subsequently cleaved at a single point to produce two chains: a heavy (H) chain (~100 kDa) and a light (L) chain (~50 kDa), linked through a disulfide bond [8]. The C-terminal part of the H chain (H_C) is responsible for neurospecific binding at the presynaptic membrane of the neuromuscular junction [9,10], while the N-terminal fragment of the H chain (H_N) plays a critical role in translocation of the catalytic L chain across the vesicle membrane into the cytosol [11]. H_C demonstrates high affinity for glycolipid receptors on the neuronal surface mainly from a ganglioside series [12]. TeNT and some of the BoNTs show a preference for ganglioside G_{T1b} [13–16]. However the interaction between this H_C and the presynaptic nerve ending is proposed to also occur through an as yet unidentified protein receptor [17]. It is assumed that differences in this protein receptor could be the reason for alternate localization of TeNT and BoNTs. The L chain, which belongs to a zinc-dependent protease family [18], catalyzes proteolysis of a specific, single protein involved in synaptic vesicle membrane fusion at a unique site inside the neuronal cytosol [19]. The result of this cleavage is inhibition of neurotransmitter release, leading to paralysis [20].

Although TeNT is effective on several types of synapses leading to spastic paralysis, while BoNTs act on the cholinergic nerve endings causing flaccid paralysis, these two subfamilies of neurotoxins exhibit significant sequence similarity and structural relatedness [21–23]. Two independently solved crystal structures of H_C of TeNT revealed the general architecture of the receptor binding domain [24,25]. This domain comprises two subdomains: the N-terminal subdomain exhibiting a jelly roll motif and the C-terminal subdomain forming a β-trefoil motif. The C-terminal subdomain of H_C is responsible for ganglioside binding; moreover, the approximate location of the ganglioside binding site within the C-terminal subdomain has been implicated by many experimental studies [26–29]. Sequence similarity for clostridial neurotoxins within this subdomain is very low, as is typical for proteins exhibiting the β-trefoil fold [30,31]. In this region, all previously reported alignments of clostridium neurotoxins show some obvious in-

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0014-5793/00/$20.00
PII: S0014-5793(00)01954-2

consistencies with the crystal structures of H_C TeNT, as well as the general architecture of the β-trefoil fold [22–24]. Consequently, we re-examined the alignment of the clostridial neurotoxin sequences in the β-trefoil subdomain with due consideration of sequence constraints imposed by this fold, and the comparative modeling studies based on the TeNT structure.

After these modeling studies had been completed, two experimental crystal structures of intact BoNT/A were reported [32,33]. This enabled verification of our proposed alignment and provided a very useful test for validation of the applied theoretical methods and modeling strategy. Taking into account structural positions of the key residues shown in previous experimental studies to be responsible for the ganglioside binding in both TeNT and BoNT/A, we re-examined the location of the putative ganglioside binding pocket within the C-terminal subdomain of H_C. It has now been shown by a crystallographic study of H_C TeNT complexed with different carbohydrate units that this pocket is able to bind lactose [34]. Our sequence-to-structure alignment for the clostridial neurotoxin family within the β-trefoil subdomain provides annotation at the level of individual residues of this putative ganglioside binding site in different serotypes of BoNT.

2. Materials and methods

2.1. Analyzing structural determinants of the β-trefoil subdomain

To produce an alignment consistent with the general architecture of the β-trefoil fold, we first identified 12 regions in the TeNT structure that are structurally conserved in this fold. These structurally conserved regions were previously determined from the superposition of the molecular structures of 13 different β-trefoil domains from proteins sharing very little sequence similarity [31], namely: ricin, abrin, acidic fibroblast growth factor, basic fibroblast growth factor, hisactophilin, interleukin 1 receptor antagonist protein, interleukin 1β, winged bean albumin 1, Kunitz trypsin inhibitor and amaranthin. The 12 conserved structural motifs of the β-trefoil fold containing 60 residues include six β-strands forming a barrel and six β-strands forming hairpins that close the bottom of the barrel. The major sequence requirements of this fold are large or medium size hydrophobic residues at 18 buried sites [30]: 12 residues from barrel strands which form the central and bottom layers, and six residues from hairpins that pack against the bottom layer. However, as shown more recently [31], residues involved in forming the central layer are much less restricted in terms of the amino acid type, reducing the number of conserved sites requiring exclusively bulky hydrophobic residues to 12.

2.2. Generating sequence-to-structure alignment

Taking into account the high sequence divergence of clostridial neurotoxins in the C-terminal part of H_C, we applied a modeling strategy that combines classical sequence alignment techniques with homology modeling studies. Initially, the CLUSTAL W program [35] was used to generate multiple sequence alignments of the C-terminus fragment of H_C of clostridial neurotoxins. Sequences of BoNT/C and BoNT/D were then excluded from the consideration, as they were too dissimilar from the rest of the family to obtain unambiguous alignment. In the CLUSTAL W alignment process neither secondary structure nor gap penalty masks were used. Opening and extension gap penalties were systematically changed to decide where the conserved parts of the alignment are located. This procedure revealed very stable parts of the alignment spanning eight structurally conserved regions of the TeNT β-trefoil structure: Tyr1128–Pro1132, Lys1138–Leu1142, Lys1174–Lys1178, Ile1196–Val1200, His1207–Tyr1211, Gly1266–Thr1270, Asp1282–Ala1286, and Asp1302–Val1306, with bulky hydrophobic residues at appropriate positions. For the remaining four structurally conserved regions in the TeNT β-trefoil structure: Asp1147–Leu1151, Arg1223–Val1227, Lys1239–Val1243, Val1253–Leu1257, where sequence similarity between BoNTs and TeNT was negligible, we considered several variants of the alignment. Taking into account the conservation of large/medium size hydrophobic amino acids at the 12 specific positions in β-trefoil fold enabled us to reduce the number of possible variants of the alignment. The remaining alignment variants were tested using the comparative modeling technique. For these cases 3D molecular models of the conserved β-trefoil core were built for all the considered serotypes of BoNT with the homology module of InsightII program package (MSI Inc., San Diego, CA, USA) using the TeNT structure [25] (PDB code: 1A8D) as a template. Positions of the conserved TeNT backbone atoms remained unchanged in the modeling procedure. Side chain conformations were generated either manually or using the SCWRL program with the backbone conformation-dependent side chain rotamer library [36]. Models were then subjected to detailed evaluation, mainly by visual inspection of the molecular structures to detect improper packing of residues, including buried unpaired donors/acceptors or exposed hydrophobic side chains. In addition, a detailed analysis was performed on the side chain packing in the core built from 18 mainly hydrophobic residues (amongst them 12 exclusively bulky hydrophobic residues) required for integrity of the β-trefoil fold. This allowed us to exclude molecular models with cavities or steric clashes that could not be removed with the rotamer search procedure, indicating either too loose or too tight packing, respectively. Such a 3D evaluation procedure enabled selection of the best alignment for the structurally conserved regions possessing very low sequence similarity. In the loop regions, where no sequence similarity was detected, the multiple sequence alignment was corrected manually to maximally preserve the interactions observed in the TeNT structure.

3. Results and discussion

3.1. Structure-based alignment for the clostridium neurotoxin family

Alignment of the target protein sequence with the template structure remains the single most significant step in the comparative modeling of protein structure [37]. Such alignments are particularly error-prone in cases of low sequence similarity. One of the major goals of this study was to investigate whether a comparative modeling approach, combined with careful consideration of 3D constraints, would result in structurally correct alignments for the clostridium neurotoxin family. Ten sequences of BoNT: BoNT/A, BoNT/A (*Inf*), BoNT/B (*GpI*), BoNT/B (*GpII*), BoNT/E, BoNT/E (*buty*), BoNT/F (*GpI*), BoNT/F (*GpII*), BoNT/F (*bara*), BoNT/G were aligned against the C-terminal fragment of H_C of TeNT. The percentage of identical residues within the β-trefoil domain, when making pairwise comparisons between TeNT and BoNT sequences, ranged from 30% to as low as 15%. Taking into account the general sequence requirements of the β-trefoil fold, and the available structural information for TeNT, the initial alignment was improved in regions where classical sequence-based algorithms were unable to detect any sequence similarity. The final alignment is shown in Fig. 1.

Recently, two crystal structures of intact BoNT/A were solved [32,33], enabling a rigorous verification of our modeling approach. As assumed in our study, structurally conserved regions of the β-trefoil fold observed in TeNT are also present in BoNT/A (Fig. 2a). These regions superimpose in both structures with the r.m.s. deviation of 0.91 Å for 60 Cα positions. The main differences between these two structures are located in loop regions, where the sequences are most divergent. However, it should be noted that Cys1234, the first residue of the structurally conserved region taking part in forming the barrel, plays a structurally different role than the corresponding Val1253 in TeNT (Fig. 2b). Specifically, the side chain of Cys1234 is not involved in forming the central layer of the hydrophobic barrel core, but forms a disulfide

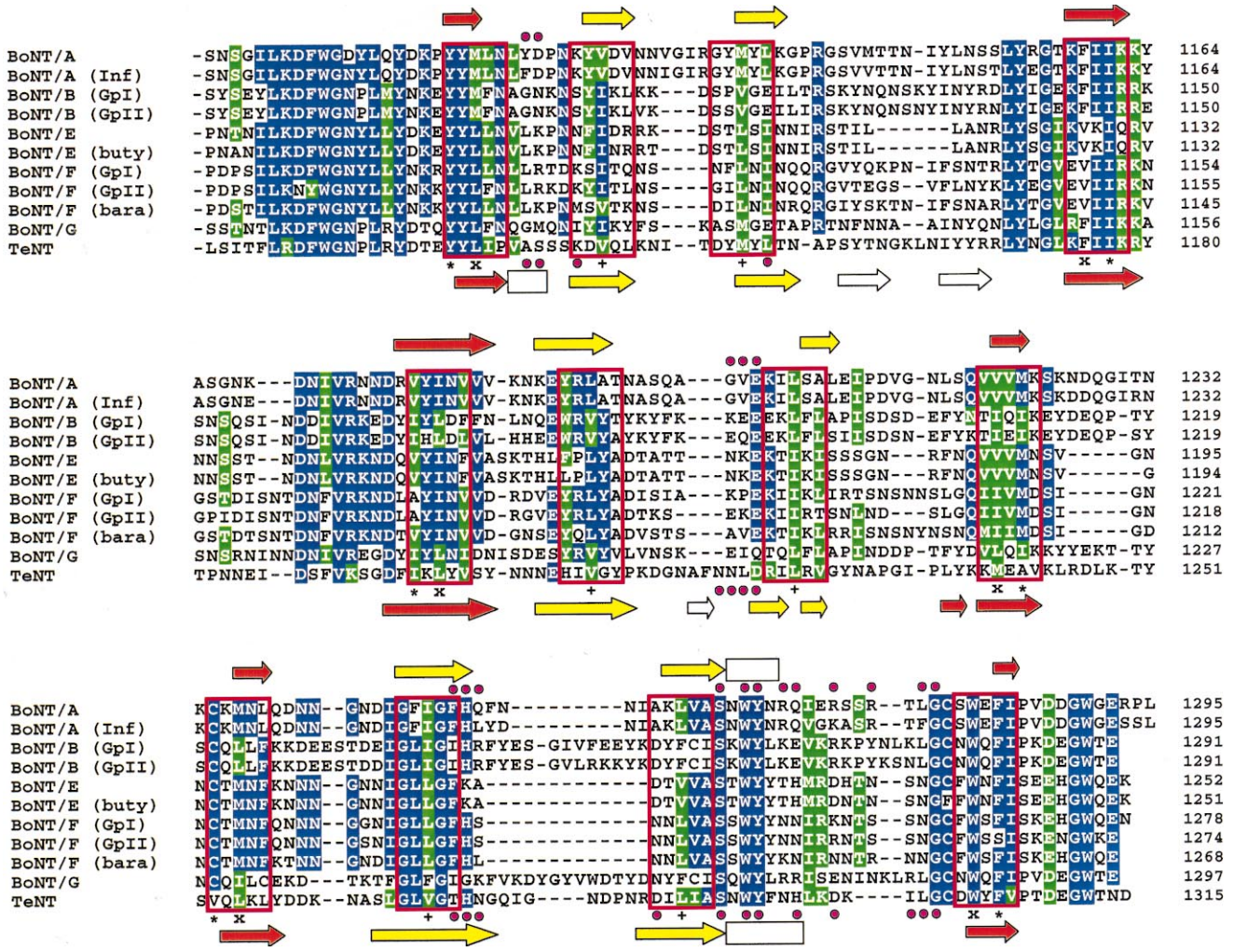

Fig. 1. Sequence alignment of TeNT and different serotypes of BoNT in the β-trefoil region. Conserved residues shared by at least 60% of all sequences are highlighted in blue (identical) and green (similar). Locations of the secondary structure elements in TeNT and BoNT/A, as defined by DSSP [42], are marked beneath and above the sequences, respectively; β-strands are represented as arrows, and α-helices as rectangles. Colored β-strands participate in either forming the barrel (brown) or closing the barrel (yellow). Twelve structurally conserved regions common to the β-trefoil fold are enclosed in red rectangles and 18 key structural positions for this fold are denoted by * (central layer), × (bottom layer) and + (hairpin closure, which packs against the bottom layer). Positions of amino acids forming the putative ganglioside binding pocket in the TeNT and BoNT/A structures are indicated by magenta dots beneath and above the sequences, respectively. Sequence numbering for the clostridial neurotoxins includes the N-terminal Met residue, except for BoNT/A, where numbering is as in the crystal structure [32].

bridge with Cys1279. This observation is consistent with the revised β-trefoil sequence requirements [31], indicating that this position is much less restricted in the choice of residue type. The role of Cys1234 is taken over by the adjacent Lys1235, which supports hydrophobic interactions in the core with the aliphatic portion of its side chain. The corresponding pairs of cysteine residues also occur in BoNT/B, BoNT/E, BoNT/F, and BoNT/G, where similar disulfide bonds are most likely formed as well.

The sequence-to-structure alignment of BoNT/A and TeNT obtained in this study is in complete agreement with the structural superposition of these two neurotoxins. This agreement extends even beyond the β-trefoil fold core into those loop regions that are structurally similar in BoNT/A and TeNT. Consequently, at the very low sequence similarity observed in this case, including structural information in the modeling strategy was the most important feature leading to the correct alignment. It should be noted that, within some conserved regions of the β-trefoil fold, the TeNT shares little or no similarity with the BoNT sequences, while in the same regions some similarity among BoNTs does exist. Thus the main difficulty in generating the sequence-to-structure alignment consisted of aligning the BoNT sequences with the previously solved structure of TeNT. Confirmation of the correctness of our modeling-derived alignment by the crystallographic studies of BoNT/A makes it reasonable to expect that the alignment for the remaining serotypes of BoNT is also correct. It should be emphasized that, even with the availability of the BoNT/A 3D structure, obtaining a structurally consistent alignment for the BoNT sequence family cannot be considered a trivial problem. To our knowledge, all the alignments of clostridial neurotoxins published to date have obvious errors in regions of low sequence similarity that are nonetheless structurally conserved in the β-trefoil fold [22–24]. This also applies to the alignment reported together with the crystal structure of BoNT/A [33]. This alignment is not only inconsistent with the general sequence requirements of the β-trefoil fold, but also with the superposition of the TeNT

and BoNT/A structures. This confirms previous observations that, in cases where proteins share very little sequence similarity, the classical sequence-based alignment techniques are extremely error-prone [38]. As a result, such alignments cannot provide reliable information at the level of individual residues regarding the β-trefoil subdomain in other serotypes of BoNT.

3.2. Ganglioside binding

Both biochemical and structural studies indicate that the β-trefoil subdomain of clostridial neurotoxins is involved in ganglioside binding. At the same time there is considerable confusion in the literature about the location and the physicochemical properties of this putative binding site.

Initially, based on the TeNT crystallographic structure, it was proposed that a shallow surface pocket formed partly by the C-terminal residues Val1306–Glu1310 might be responsible for ganglioside binding [24], since their deletion in truncation studies completely abolished ganglioside binding [26]. Recently, more direct experiments identified a different region involved in ganglioside binding. A study using novel ganglioside photoaffinity ligand implicated His1293 in TeNT [27]. A similar region in BoNT/E containing His1228, which corresponds to His1293 in TeNT, was also identified by using monoclonal antibodies [28]. In addition, it was shown that ganglioside quenches tryptophan fluorescence, suggesting that a tryptophan must be part of the ganglioside binding site in BoNT/A [29]. The X-ray structure of BoNT/A revealed that only one of the three tryptophan residues in the C-terminal region of the β-trefoil subdomain, Trp1265, is exposed to solvent [33]. Both Trp1265 in BoNT/A (Trp1289 in TeNT) and His1293 in TeNT (Gln1269 in BoNT/A) are located close to one another at *i* and *i*+4 positions of the same α-helix, but are on the opposite side of the β-trefoil subdomain relative to the C-terminal residues of TeNT (Val1306–Glu1310) removed in the truncation study [26]. This apparent discrepancy between two sets of experimental data can be resolved if removal of Val1306, the terminal residue of the last strand of the β-trefoil core, destroys the integrity of the whole β-trefoil subdomain, including the ganglioside binding site, as suggested by Emsley et al. [34].

Surprisingly, in spite of the identification of His1293 in TeNT and Trp1265 in BoNT/A as part of the putative ganglioside binding site, there has been considerable confusion regarding surface location, shape and electrostatic properties of this site. A discussion by Lacy and Stevens [33] regarding the deep, positively charged interdomain cleft in BoNT/A, left others [34] thinking that this cleft was being proposed as the ganglioside binding site. The cleft in the subdomain interface is formed partly by a loop (Lys1244–Ser1252 in TeNT and Ser1224–Lys1233 in BoNT/A), whose conformation differs significantly in TeNT and BoNT/A structures. As proposed by Lacy and Stevens [33], the large difference in shape and electrostatic properties of this cleft might contribute to alternate localization of these two toxins. If so, we suggest that the role of this cleft is to interact with the still unidentified protein receptor rather than the ganglioside.

The two key residues implicated in ganglioside binding in both structures are located on the rim of a shallow surface pocket, which is distinct from the interdomain cleft (Fig. 3a). Residues forming this pocket in TeNT and BoNT/A structures are marked in Fig. 1. Positions of most of these TeNT and BoNT/A residues are structurally conserved, as they come from conserved secondary structure elements, or from those parts of loops which are also structurally similar. These structurally equivalent residues superimpose closely, with the r.m.s. deviation of 0.68 Å for their Cα positions.

The importance of this pocket has now been confirmed by a crystallographic study showing that this region in the TeNT structure binds lactose [34]. Furthermore, the orientation of the bound lactose would allow accommodating the rest of the ganglioside structure without any steric hindrance (Fig. 3b). Although the same study identified other possible binding sites within the β-trefoil subdomain for several different gan-

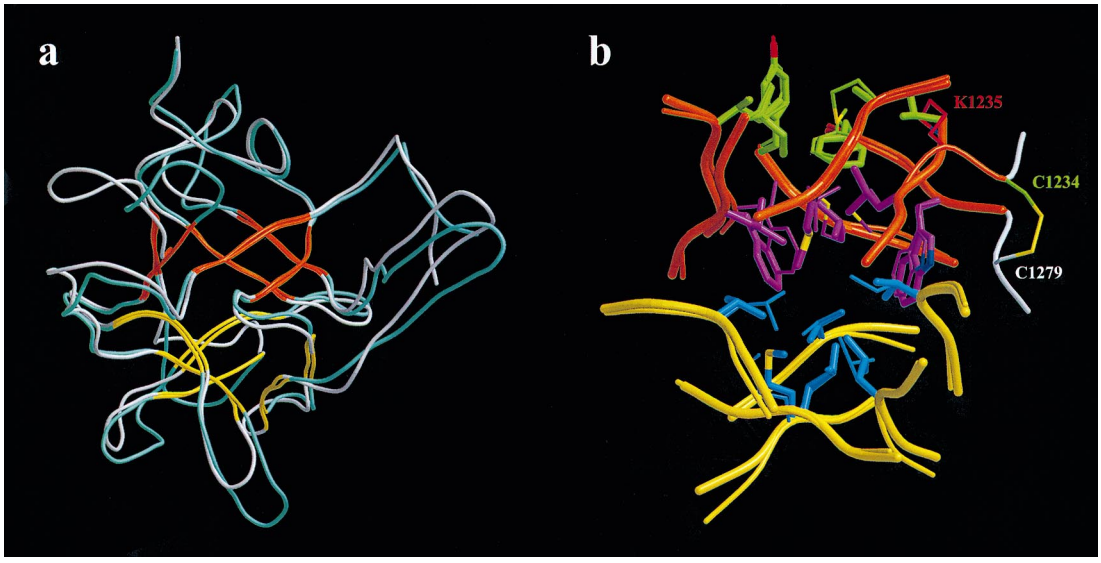

Fig. 2. Comparison of the TeNT and BoNT/A β-trefoil subdomains, superimposed within structurally conserved regions. a: Entire β-trefoil region in TeNT (cyan) and BoNT/A (white). Structurally conserved regions common to this fold are colored brown and yellow in both structures for the barrel and hairpins, respectively. b: Enlarged β-trefoil core for TeNT (thick) and BoNT/A (thin). Side chains of 18 key residues forming the core are shown as sticks, including residues from the central layer (green), bottom layer (magenta) and hairpin closure (blue). Labeled are a pair of cysteine residues and lysine that contribute to the unusual packing in the β-trefoil core of BoNT/A.

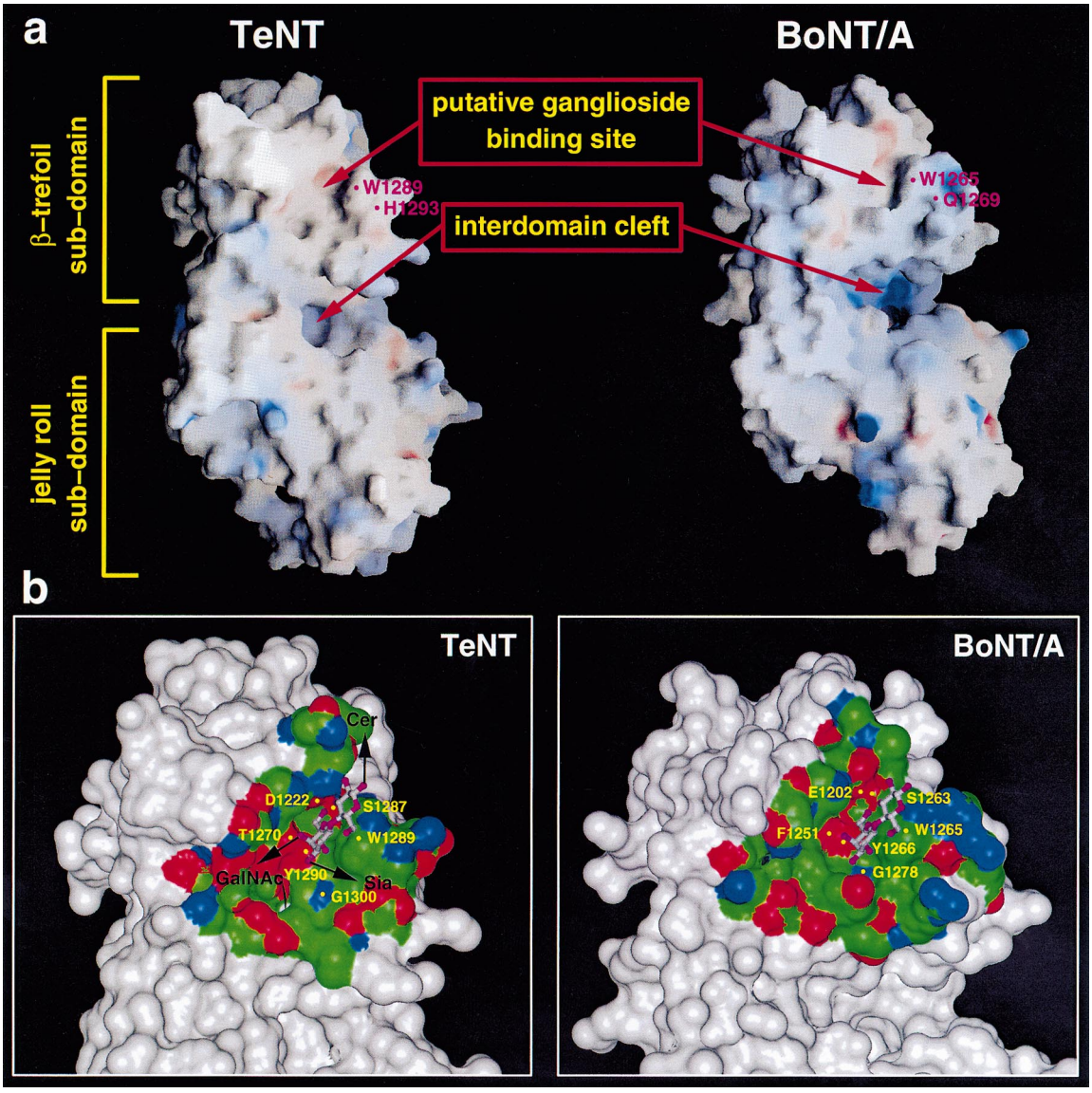

Fig. 3. Comparison of binding domains for TeNT and BoNT/A. a: GRASP [43] representation of the molecular surfaces colored by electrostatic potential: positive in blue and negative in red. b: Enlarged molecular surfaces in the region of the putative ganglioside binding site. The putative binding site is colored by atom type: red (oxygen), blue (nitrogen) and green (carbon). Labeled are residues contributing to the conserved features of the putative ganglioside binding pocket. Bound lactose unit is shown as balls and sticks. Black arrows indicate positions of the remaining parts of ganglioside receptor: cer, ceramide; sia, sialic acid; GalNAc, *N*-acetylgalactosamine. Lactose in the BoNT/A structure was modeled based on the lactose–TeNT complex [34].

glioside subunits, it remains to be determined if these subunits actually bind in the same manner when they are a part of the intact ganglioside receptor. Taking into account the agreement between structural and biochemical data, we suggest that the lactose binding site plays the most important role in the ganglioside recognition and binding.

In contrast to the interdomain cleft, the shape and the electrostatic properties of the putative ganglioside binding site in both toxins appear to be much more similar (Fig. 3). In particular, in both toxins the surface of this pocket does not have any significant electrostatic charge, but the bottom of the pocket possesses a similar hydrophilic patch. This highly hydrophilic part of the surface is formed in both toxins by pairs of the structurally equivalent residues from the conserved secondary structure elements. In TeNT this includes side chain oxygen atoms of Asp1222, Ser1287, Tyr1290 (Glu1202, Ser1263, Tyr1266 in BoNT/A), the main chain carbonyl group of Thr1270 (Phe 251 in BoNT/A) and the main chain amide group of Gly1300 (Gly1278 in BoNT/A). All these polar atoms make hydrogen bonds with oxygen atoms of the galactose ring in TeNT [34]. This finding suggests the importance of this conserved hydrophilic surface pattern at the bottom of the pocket, which seems to play the key role in binding of the internal galactose ring of ganglioside. Another important common feature of this putative ganglioside binding site in TeNT and BoNT/A is a hydrophobic wall formed by a key tryptophan residue. As shown in the TeNT structure [34] this Trp1289 plays a critical role in maintaining hydrophobic interactions between the toxin and the ganglioside galactose ring. Similar stacking interactions between a tryptophan side chain and a galactose ring have been observed both in cholera toxin [39] and heat-labile enterotoxin [40,41] structures com-

plexed with part of the ganglioside receptor, arguing for the importance of such interaction in recognition and binding.

Only a few residues in this putative ganglioside binding pocket, including Ser1287, Trp1289, Tyr1290 and Gly1300 in TeNT, are absolutely conserved throughout the clostridial neurotoxin family (without consideration of BoNT/C and BoNT/D). In addition to these four fully conserved residues, Asp1222 in TeNT is replaced by glutamic acid in most of BoNT serotypes. Interestingly, all these residues were found to interact with the galactose unit. Consequently, the conservation of these five residues seems to be, as discussed above, a common recognition pattern responsible for galactose binding.

Acknowledgements: This work was performed under the auspices of the United States Department of Energy by Lawrence Livermore National Laboratory under contract number W-7405-Eng-48, with additional support from Warsaw University (K.G., BST 622/BF and B.L., BST 681/ICM). The authors would like to thank Dr. Rod Balhorn for suggesting this study and insightful comments, and Dr. D. Shugar for critical reading of the manuscript.

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