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Structural insights into the functional role of the Hcn sub-domain of the receptor-binding domain of the botulinum neurotoxin mosaic serotype C/D

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Abstract

Botulinum neurotoxin (BoNT), the causative agent of the deadly neuromuscular disease botulism, is the most poisonous protein known for humans. Produced by different strains of the anaerobic bacterium *Clostridium botulinum*, BoNT effects cellular intoxication via a multistep mechanism executed by the three modules of the activated protein. Endocytosis, the first step of cellular intoxication, is triggered by the ~50 kDa, heavy-chain receptor-binding module (HCR) that is specific for a ganglioside and a protein receptor on neuronal cell surfaces. This dual receptor recognition mechanism between BoNT and the host cell's membrane is well documented and occurs via specific intermolecular interactions with the C-terminal sub-domain, Hcc, of BoNT-HCR. The N-terminal sub-domain of BoNT-HCR, Hcn, comprises ~50% of BoNT-HCR and adopts a β -sheet jelly roll fold. While suspected in assisting cell surface recognition, no unambiguous function for the Hcn sub-domain in BoNT has been identified. To obtain insights into the potential function of the Hcn sub-domain in BoNT, the first crystal structure of a BoNT with an organic ligand bound to the Hcn sub-domain has been obtained. Here, we describe the crystal structure of BoNT/CD-HCR determined at 1.70 Å resolution with a tetraethylene glycol (PG4) moiety bound in a hydrophobic cleft between β -strands in the β -sheet jelly fold roll of the Hcn sub-domain. The PG4 moiety is completely engulfed in the cleft, making numerous hydrophobic (Y932, S959, W966, and D1042) and hydrophilic (S935, W977, L979, N1013, and I1066) contacts with the protein's side chain and backbone that may mimic *in vivo* interactions with the phospholipid membranes on neuronal cell surfaces. A sulfate ion was also observed bound to residues T1176, D1177, K1196, and R1243 in the Hcc sub-domain of BoNT/CD-HCR. In the crystal structure of a similar protein, BoNT/D-HCR, a sialic acid molecule was observed bound to the equivalent residues suggesting that residues T1176, D1177, K1196, and R1243 in BoNT/CD may play a role in ganglioside binding.

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Keywords

botulinum neurotoxin; endocytosis; botulism; protein X-ray crystallography; ganglioside

1. Introduction

Botulinum neurotoxins (BoNTs), the etiological agents responsible for the disease botulism, are the most potent protein toxins for humans with a median lethal dose (LD₅₀) below 1 ng per kg of body weight [1]. The extreme potency of BoNTs is due to their ability to inhibit neurotransmitter release at unmyelinated regions of nerve terminals resulting in flaccid muscular paralysis [2]. While natural cases of human botulism are rare, botulism outbreaks in livestock are common and result in significant economic losses worldwide [3]. A greater concern is the potential use of BoNTs as an agent of bioterrorism. The Centers for Disease Control and Prevention (CDC) in the United States lists BoNT as one of the six “Class A” highest-risk threat agents for bioterrorism [4].

Botulism neurotoxins are produced by different strains of the anaerobic bacterium *Clostridium botulinum* with the immunologically distinct serotypes A, B, E, and F causing human botulism and the serotypes C and D primarily affecting animals [5–7]. Animals are also susceptible to two BoNT mosaic proteins that are essentially hybrids of the C and D serotypes, BoNT/CD (~two-third BoNT/C and ~one-third BoNT/D) and BoNT/DC (~one-third BoNT/C and ~two-third BoNT/D). Each BoNT isoform is initially translated as an ~150 kDa single chain polypeptide that is proteolytically activated and assembled into a molecule with a N-terminal ~ 50 kDa light chain (LC) and a C-terminal ~ 100 kDa heavy chain (HC) held together with a disulfide bond [8, 9]. This mature toxin consists of three modules with distinct functions. The LC polypeptide is a zinc-dependent, sequence specific, endopeptidase responsible for the inactivation of host proteins, SNAP-25 (synaptosome-associated protein of 25 kDa) and/or synaptobrevin, essential for neurotransmitter release. The N-terminal half of the HC polypeptide, Hn, is the translocation domain responsible for forming a transmembrane channel in endosomes to translocate the LC polypeptide into the cytosol. The C-terminal half of the HC polypeptide is the receptor-binding domain (HCR) responsible for neuronal cell specificity and endocytosis initiation.

The receptor-binding module of BoNTs exploits the synaptic vesicle recycling pathways to recognize and gain entry into neuronal cells. With BoNT serotypes A, B, E, F, and G this exploitation involves a dual host-receptor mechanism with a synaptic vesicle protein and ganglioside [10]. The co-receptor protein is synaptogamin I or II for serotypes B and G [11, 12] and synaptic vesicle protein 2 for serotypes A, D, E, and F [13–16]. A protein co-receptor has not been identified for BoNT serotypes C. The co-receptor gangliosides are GT1b, GD1b, and GD1a for serotypes A, B, C, and F, GT1b and GD1a for serotype E, and all gangliosides for serotype G [16–19]. These BoNT-ganglioside interactions are facilitated by a SxWY motif located in the C-terminus of the HCR domain [20]. Serotype D lacks a SxWY motif but has been shown to bind gangliosides [14, 21] as well as phosphatidylethanolamine [22].

The ~ 50 kDa HCR domain contains two structural sub-domains of approximately equal size, Hcc and Hcn. Both the protein and ganglioside receptor sites in HCR have been observed only in the Hcc sub-domain in all BoNT serotypes [20]. While it is suspected that the Hcn subdomain modulates interactions with the surface of neuronal cells, perhaps through interactions with sphingomyelin-enriched membrane regions [23], no unambiguous function for the Hcn subdomain has yet been identified. To obtain insights into the potential function of the Hcn subdomain, we have determined the crystal structure of the HCR

domain of BoNT/CD (S867-E1280) at 1.70 Å resolution with tetraethylene glycol (PG4), a moiety that mimics the hydrophobic fatty acid tails of phospholipids, bound to the Hcn sub-domain. To the best of our knowledge this represents the first BoNT crystal structure with an organic ligand observed associated with the Hcn sub-domain. Also observed in the new BoNT/CD crystal structure was a sulfate ion bound to the putative ganglioside-binding site in the Hcc sub-domain that may provide further insights into the ganglioside-binding mechanism.

2. Materials and methods

2.1 Protein expression and purification

Details of the expression and purification of the receptor binding domain of *C. botulinum* (S867-E1280, BoNT/CD-HCR) have previously been described [22, 24]. Briefly, BoNT/CD-HCR was expressed using a codon-optimized cDNA in *Escherichia coli* and purified using Ni-NTA, ion exchange, and size exclusion chromatography.

2.2 Crystallization and structure determination

Purified BoNT/CD-HCR was concentrated to ~ 5 mg/mL and screened for crystal growth by vapor diffusion using 2 µL of protein and 2 µL of precipitant from commercial crystallization kits (Hampton Research, Aliso Viejo, CA and Emerald BioSystems, Bainbridge Island, WA) [20]. Diffraction quality crystals were obtained at room temperature with mother liquor composed of 1.6 M (NH₄)₂SO₄, 2% PEG 400, and 0.1 M Hepes, pH 7.5. To cryoprotect the crystals, crystals were transferred in a stepwise fashion into cryoprotectant solutions containing increasing concentrations of glycerol and vitrified in liquid nitrogen. X-ray diffraction data was collected at the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory on beamline 5.0.1 with an ADSC Q315R detector. Data was processed using *DENZO* and integrated intensities scaled using the *SCALEPACK* program from the *HKL-2000* software package [25]. Original phasing for the BoNT/CD-HCR crystal grown under these conditions was obtained by molecular replacement using *Phaser* of the *CCP4* suite of programs [26] and the first reported crystal structure of BoNT/CD-HCR (PDB ID: 3PME) for the molecular search template [22]. Both *Coot* [27] and *CCP4* [24] were used to refine the model and *Molprobity* [28] was used to validate the model. Coordinates and structure factors for the new BoNT/CD-HCR structure were deposited in the Protein Data Bank (PDB) with accession number 4F83.

3. Results and discussion

3.1 The BoNT/CD-HCR structure

The data collection and structural refinement statistics summarized in Table 1 indicate the final model was a quality representation of the structure for BoNT/CD-HCR. The resolution of this new BoNT/CD-HCR structure, 1.70 Å, was slightly lower than the 1.56 Å resolution reported for the first crystal structure for BoNT/CD-HCR (PDB ID 3PME) [22]. However, unlike the original structure that contained two sulfate ions, one glycerol molecule, and one protein unit in the asymmetric unit, the new structure contained three sulfate ions, two glycerol molecules, and three tetraethylene glycol (PG4) moieties along with the single protein unit in the asymmetric unit. Electron density was modeled into the entire protein except for the final four histidines on the C-terminal polyhistidine affinity tag and residues S929 - I931 in the Hcn subdomain. By contrast, in the original structure electron density could not be modeled into the final two histidines at the C-terminal, S867, and residues N1218 and K1219 in the Hcc subdomain [22].

The new (4F83) and original (3PME) BoNT/CD-HCR structures are similar, as illustrated in the superposition in Figure 1, with a backbone root mean square deviation (RMSD) of 0.7 Å over 409 aligned Cα atoms. Indeed, the DALI Z-score [29] for the pair of structures is 60.7. The Hcn sub-domain (S867-S1072) adopts a β-sheet jelly roll fold and the Hcc sub-domain (L1085-E1280) is organized into a β-trefoil fold with a helix (N1073-I1084) linking the two sub-domains. These general features have been observed in all other BoNT-HCR structures [20]. The structural differences that exist between the new and original BoNT/CD-HCR structures are subtle variations in the length of corresponding α-helices and β-strands and minor shifts of some loops. While the majority of these subtle differences likely have no functional implications, a few arise due to additional ligands bound to BoNT/CD-HCR in the new structure.

3.2 Potential membrane binding site in the Hcn sub-domain

In addition to the precipitant conditions reported to crystallize and solve the original structure for BoNT/CD-HCR (20% (w/v) PEG 8000, 10% (w/v) isopropanol, 0.2 M (NH₄)₂SO₄, and 0.1 M Hepes, pH 7) [22], crystal were also obtained under various other conditions. X-ray diffraction data was collected on some of these differently generated crystals and electron density maps were examined for additional electron density that might signal the presence of new ligands bound to BoNT/CD-HCR. For the crystals grown with 1.6 M (NH₄)₂SO₄, 2% PEG 400, and 0.1 M Hepes, pH 7.5, substantial regions of continuous electron density were observed in the Hcn sub-domain that warranted the full refinement of the XRD data. Considering the protein buffer (Tris and NaCl), precipitants ((NH₄)₂SO₄, PEG 400, and Hepes) and cryoprotectant (glycerol), this electron density was best modeled as a tetraethylene glycol moiety (PG4), a part of PEG 400. Isothermal titration calorimetry experiments show that PEG 400 weakly binds to BoNT/CD-HCR with a K_a in the 10² to 10³ M⁻¹ range (data not shown). As illustrated in Fig. 2A, this PG4 moiety adopts a 'bow-like' conformation, buried in a hydrophobic cleft between β-strands (β6, β8, β9, β11 and β16) in the jelly roll fold of the Hcn sub-domain. A loop (Y928-N934) partially covers PG4 that appears to be flexible because electron density could not be confidently modeled for three (S929-I1131) of the seven residues (Fig. 1B). This flexibility may be due to the association with PG4 because the same residues were confidently modeled in the original BoNT/CD-HCR structure. Numerous hydrophobic (Y932, S959, W966, and D1042) and hydrophilic (S935, W977, L979, N1013, and I1066) interactions, summarized schematically in Figure 2B, hold PG4 in position. The hydrophilic interactions include potential hydrogen bonds between the proton of the terminal PG4 hydroxyl groups, O1 and O5, with the main chain carbonyl group of Y932 and D1042, respectively, the PG4 ether (O4) and hydroxyl (O5) oxygens with the side chain amide (NE1) proton of W966, and the terminal PG4 O5 hydroxyl proton with the side chain hydroxyl oxygen of S959. Mapping of the nine BoNT/CD-HCR residues (blue triangles) that make contact with PG4 onto the sequence alignment (Fig. 3) of the Hcn sub-domain of BoNT/CD and BoNT serotypes A through F reveals that six out of the nine residues (Y932, W966, W977, L979, N1013, and I1066) are highly conserved. Perhaps such a conservation of PG4 binding residues in all BoNT serotypes is due to a common motif in the Hcn sub-domain that assists neuronal cell recognition.

It has been suggested that the Hcn sub-domain modulates interactions with the surface of neuronal cells, perhaps through interactions with sphingomyelin-enriched microdomains of the plasma membrane [23]. Additional support for this hypothesis is the observation that BoNT/C-HCR binds phosphoinositides [30], lipids that mimic sphingomyelin. The long chain, repeating -O-C-C-O-backbone of PG4 mimics the alkyl chain of both the fatty acid and sphingosine group of sphingomyelin. Hence, the observed PG4 interactions with the Hcn sub-domain of BoNT/CD-HCR may mimic *in vivo* interactions of the botulinum neurotoxin with neuronal cell surfaces. If true, this feature may be universal to all BoNT

serotypes because the Hcn sub-domain is highly conserved in all BoNTs [31], forming a similar β -sheet jelly roll fold in all BoNT structures and, as illustrated in Figure 4A, adopting a similar orientation relative to the Hcc sub-domain [32–37].

On the basis of sequence (27.5 % identical) and structural similarity of the Hcn subdomain of BoNT/A to proteins that bind phosphatidylinositol monophosphate, it was predicted that residues K876 – N929 comprised the membrane interaction site of Hcn in BoNT/A [21]. This region contained a number of basic arginine and lysine residues that were candidates for association with the phosphate group of sphingomyelin. In BoNT/CD the corresponding region is K872 – N925, residues that were not observed to associate with PG4 (Fig. 4B). However, this predicted sphingomyelin-binding region of Hcn, colored red in Figure 4B, is next to the bound PG4. Sphingomyelin is a larger molecule than the 13 heavy-atom long PG4 moiety and contains hydrophilic functional groups. If the PG4-binding cleft is related to sphingomyelin-binding, it may only reflect a part of the sphingomyelin-protein interaction surface, for example, association with sphingosine or fatty acid alkyl group normally buried in the plasma membrane. Regardless of whether both, or only one, of either the PG4-binding pocket or the predicted membrane binding surface (K872 – N925) are involved in membrane binding, both regions are opposite the predicted ganglioside binding region in the Hcc sub-domain (Fig. 4B). Consequently, as suggested by Muraro *et al.* [23], joint ganglioside and sphingomyelin binding would effect a reorientation of the Hcn sub-domain relative to the Hcc sub-domain that may serve to swing the N-terminal half of the HC polypeptide, Hn (translocation domain), into position for insertion into the lipid bilayer.

3.3 Potential membrane binding site in the Hcc sub-domain

Neurotoxicity is dependent upon ganglioside binding for both BoNT/C and BoNT/D [16, 21]. While BoNT/CD does not have an SxWY ganglioside binding motif, BoNT/CD-HCR has been shown to bind to synaptosomes [38] and phosphatidylethanolamine [22]. Insights into the possible binding motif in BoNT/D was provided by a co-crystal structure of BoNT/D-HCR with sialic acid, a component of membrane gangliosides, showing the sialic acid bound in a shallow groove formed by the loops Y1235-Y1246, E1114-V1117, and R1170-L1191 [21]. The residues forming these loops in BoNT/D are highly conserved in the amino acid sequence of BoNT/CD suggesting these corresponding residues may be involved in ganglioside binding by BoNT/CD. However, previous comparison of the apo-structures of BoNT/CD-HCR and BoNT/D-HCR show that the BoNT/CD loops Y1239-Y1250 and K1118-I1121 shift ~ 2 Å away from the protein core relative to the equivalent BoNT/D loops Y1235-Y1246 and E1114-V1117 (Fig. 5) [22]. Furthermore, the middle of BoNT/D loop R1170-L1191 is disordered whereas the equivalent region in BoNT/CD is well ordered. Such structural differences in the putative ganglioside-binding region in the apo-structures of BoNT/CD-HCR and BoNT/D-HCR raised questions about the ability of this motif in BoNT/CD to bind gangliosides; a conclusion indirectly supported by unsuccessful attempts to co-crystallize BoNT/CD-HCR bound to sialic acid. Evidence in support of the involvement of BoNT/CD loops Y1239-Y1250, K1118-I1121, and R1174-L1195 in ganglioside binding is the observation of a sulfate bound in this region in the new BoNT/CD crystal structure reported here.

As shown globally in Figure 1 and locally in Figure 6A and 6B, a sulfate ion is bound within the loops of Y1239-Y1250, K1118-I1121, and R1174-L1195, interacting with residues T1176, D1177, K1196, and R1243, the same four equivalent residues that make contact with sialic acid in the BoNT/D-HCR/sialic acid complex (T1172, D1173, K1192, and R1239, respectively). Three out of the four sulfate oxygens are in position to potentially form multiple hydrogen bonds with the main chain amide proton of T1176 and D1177, the side chain amino protons of K1196, and the side chain guanidinium protons of R1243 (Fig. 6B). This potential hydrogen bonding pattern with the sulfate ion and BoNT/CD is nearly

identical to the potential hydrogen bonding pattern with the sialic acid molecule and BoNT/D-HCR [21]. While sialic acid and sulfate ions are chemically different, the observation that the same corresponding residues in the same corresponding regions in BoNT/D and BoNT/CD bind these different ligands in very similar ways suggests that this region may serve a common function - binding gangliosides.

4. Conclusions

The amino acid sequence of the Hcn sub-domain is highly conserved in all BoNTs serotypes [31] and adopts a similar three-dimensional fold [32–37]. Such a correlation suggests a common biological function for the Hcn sub-domain and it has been proposed that this common function is the modulation of interactions with the surface of neuronal cells [23]. The first crystal structure reported here of a BoNT with an organic ligand bound to the Hcn sub-domain adds to this slowly growing body of evidence in support of this hypothesis. The tetraethylene glycol moiety bound in a hydrophobic cleft between β -strands in the β -sheet jelly roll fold of the Hcn sub-domain may mimic *in vivo* interactions with the phospholipid membranes on neuronal cell surfaces. Also observed in the BoNT/CD-HCR crystal structure reported here was a sulfate ion bound to the same region of the Hcc sub-domain as observed for sialic acid bound to BoNT/D-HCR. This finding provides additional evidence to support the hypothesis that loops Y1239-Y1250, K1118-I1121, and R1174-L1195 in BoNT/CD plays a role in ganglioside binding. Further studies are necessary to unambiguously identify potential interactions between BoNT/CD-HCR and the neuronal membrane and the biological role of the Hcn sub-domain in all BoNT serotypes.

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Abbreviations

BoNT	botulinum neurotoxin
HCR	receptor-binding domain
HC	heavy chain
LC	light chain
Hcc	C-terminal sub-domain of HCR
Hcn	N-terminal sub-domain of HCR
PG4	tetraethylene glycol

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Highlights

- First BoNT crystal structure with an organic ligand bound to the Hcn sub-domain.
- Botulinum-PEG 400 interactions in the Hcn sub-domain may mimic *in vivo* interactions.
- Sulfate ion bound to the putative ganglioside-binding site in the Hcc sub-domain.

Highlights

- Crystal structure of the receptor-binding domain of the botulinum C/D mosaic neurotoxin determined at 1.70 Å resolution.
- First crystal structure of the receptor-binding domain of a botulinum neurotoxin with an organic ligand bound to the Hcn sub-domain.
- Botulinum-PG4 interactions may mimic *in vivo* interactions, providing additional clues about the functional role of the Hcn sub-domain.
- Sulfate ion bound to the putative ganglioside-binding site in the Hcc sub-domain may be relevant to the ganglioside-binding mechanism.

Fig. 1. Comparison of the BoNT/CD-HCR structures. (A) Cartoon representation of the superposition of the new (green, 4F83) and original (magenta, 3PME) BoNT/D-HCR crystal structures. (B) An approximately 180° rotation of Figure 1A about the y-axis. The Hcn and Hcc sub-domains are indicated and important regions identified with arrows. The bound sulfate ion and PG4 moiety in the new BoNT/CD-HCR structure are shown in space-filling representation with carbon, oxygen, and sulfur atoms colored gray, red, and yellow, respectively.

Fig. 2. Structural details of the PG4 moiety bound to the Hcn sub-domain of BoNT/CD-HCR. (A) PG4 is illustrated in a stick representation with the carbon and oxygen atoms colored green and red, respectively, and the $2|F_o|-|F_c|$ electron-density map is shown in blue mesh contoured at 1.0σ . (B) Schematic representation of the hydrophobic contacts (arc with radiating spokes) and potential hydrogen bonds (dashed lines) between PG4 and BoNT/CD-HCR (*LIGPLOT*) [39].

Fig. 3.

Sequence alignment of the Hcn sub-domains of BoNT/CD and BoNT serotypes A through F. Identical and conserved residues are shaded red and yellow, respectively, with the numbering scheme along the top corresponding to BoNT/CD. Blue triangles identify amino-acid residues in BoNT/CD that interact with PG4 (from Fig. 2B). The figure was prepared using the online ESPript server (<http://esprict.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>).

Fig. 4.

The Hcc and Hcn sub-domains in BoNTs are similarly orientated. (A) Cartoon representation of the crystal structure of the HCR domain of BoNT/A (3BTA), BoNT/B (1EPW), BoNT/C (3N7K), BoNT/D (3OGG), BoNT/DC (3N7L), BoNT/E (3FFZ), and BoNT/F (3FUQ) superimposed upon the crystal structure of BoNT/CD (4F83). The Hcc and Hcn sub-domains are colored green and blue, respectively, with the helix linking the sub-domains colored magenta. (B) Cartoon representation of the crystal structure of BoNT/CD with PG4 and sulfate ion shown with a space-filling representation. The Hcn sub-domain is colored blue, predicted sphingomyelin-binding region in the Hcn sub-domain colored red, Hcc sub-domain colored green, predicted ganglioside-binding region in the Hcc sub-domain colored orange, and the helix linking the two sub-domains colored magenta.

Fig. 5.

The original BoNT/CD-HCR crystal structure (magenta, 3PME) superimposed upon the crystal structure of BoNT/D-HCR in complex with sialic acid (cyan, 3OBT). The structures are shown in a cartoon representation. The sialic acid bound to BoNT/D is shown in a space-filling representation with carbon, oxygen, and nitrogen atoms colored green, red, and blue, respectively. The corresponding regions in BoNT/CD that may be involved in ganglioside-binding are labeled with arrows.

Fig. 6. Structural details of the sulfate ion bound to the Hcc sub-domain of BoNT/CD-HCR. (A) The sulfate ion is shown in a space-filling representation with the $2|F_o|-|F_c|$ electron-density map shown in blue mesh contoured at 1.0σ . The four amino-acid residues that interact with the sulfate ion are labeled and shown in a stick representation. (B) Schematic representation of the potential hydrogen bonds (dashed lines) between the sulfate ion and BoNT/CD-HCR (*LIGPLOT*) [39].

Table 1

Data collection and structure refinement statistics for BoNT/CD-HCR.

Data collection	
Space group	P2 ₁
Unit-cell parameters	
<i>a</i> (Å)	40.5
<i>b</i> (Å)	112.1
<i>c</i> (Å)	50.2
$\alpha = \gamma$ (°)	90.0
β (°)	98.6
Resolution (Å)	50.0–1.70 (1.76–1.70)
Redundancy	3.7 (3.6)
<i>I</i> / σ (<i>I</i>)	24.3 (2.9)
<i>R</i> _{merge}	0.05 (0.47)
Completeness (%)	98.3 (96.9)
Structural refinement	
<i>R</i> _{work} (%)	16.9
<i>R</i> _{free} (%)	19.8
RMSD in bond length (Å)	0.016
RMSD in bond angle (°)	1.27
MolProbity analysis	
MolProbity score	1.59 (86 th) [*]
Clashscore, all atoms	8.58 (78 th) [*]
Ramachandran favored (%)	97.31
Ramachandran additionally allowed (%)	2.45
Ramachandran outlier (%)	0.24
Residues with bad bonds (%)	0.0
Residues with bad angles (%)	0.0

^{*} 100th percentile is the best and 0th percentile is the worst among structures of comparable resolution.