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$^1\text{H}^{\text{N}}$, ^{13}C , and ^{15}N Resonance Assignments of the CDTb-Interacting Domain (CDTaBID) from the *Clostridium difficile* Binary Toxin Catalytic Component (CDTa, residues 1–221)

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Abstract

Once considered a relatively harmless bacterium, *Clostridium difficile* has become a major concern for healthcare facilities, now the most commonly reported hospital-acquired pathogen. *C. difficile* infection (CDI) is usually contracted when the normal gut microbiome is compromised by antibiotic therapy, allowing the opportunistic pathogen to grow and produce its toxins. The severity of infection ranges from watery diarrhea and abdominal cramping to pseudomembranous colitis, sepsis, or death. The past decade has seen a marked increase in the frequency and severity of CDI among industrialized nations owing directly to the emergence of a highly virulent *C. difficile* strain, NAP1. Along with the large *Clostridial* toxins expressed by non-epidemic strains, *C. difficile* NAP1 produces a binary toxin, CDT (*C. difficile* transferase). As the name suggests, CDT is a two-component toxin comprised of an ADP-ribosyltransferase (ART) component (CDTa) and a cell-binding/translocation component (CDTb) that function to destabilize the host cytoskeleton by covalent modification of actin monomers. Central to the mechanism of binary toxin-induced pathogenicity is the formation of CDTa/CDTb complexes at the cell surface. From the perspective of CDTa, this interaction is mediated by the N-terminal domain (residues 1–215) and is spatially and functionally independent of ART activity, which is located in the C-terminal domain (residues 216–420). Here we report the $^1\text{H}^{\text{N}}$, ^{13}C , and ^{15}N backbone resonance assignments of a 212 amino acid, ~23 kDa N-terminal CDTb-interacting domain (CDTaBID) construct by heteronuclear NMR spectroscopy. These NMR assignments represent the first component coordination domain for a family of *Clostridium* or *Bacillus* species harboring ART activity. Our assignments lay the foundation for detailed solution state characterization of structure-function relationships, toxin complex formation, and NMR-based drug discovery efforts.

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Conflict of Interest. The authors declare that they have no conflict of interest.

Keywords

Clostridium difficile infection (CDI); CDTa; Binary toxin; CDTb-interacting domain (BID); ART (ADP-ribosyltransferase)

Biological context

Clostridium difficile is a gram-positive human and animal pathogen responsible for *Clostridium difficile* infection (CDI) and associated diseases (CDAD). In the United States, CDI is the leading cause of health care-associated infection, accounting for more than 300,000 cases and 27,000 deaths annually (Lessa et al. 2015). Typical nosocomial infections result from opportunistic *C. difficile* colonization following antibiotic-associated disruption of the normal gut flora. Complications can be mild to severe and include watery diarrhea, pseudomembranous colitis, toxic megacolon, and death. Virulence of historical *C. difficile* strains is primarily attributed to the production of two large toxins, Toxin A (TcdA) and Toxin B (TcdB), that promote inflammation and tissue damage in host epithelial cells. The past decade has seen a dramatic increase in the number and severity of CDI cases in North America and Europe, coinciding with the emergence of an epidemic isolate, NAP1 (North American pulsed-field gel electrophoresis type 1) (Perelle et al. 1997). Hypervirulence of *C. difficile* strain NAP1 is conferred through fluoroquinolone resistance, upregulation of the TcdA/TcdB locus, and the presence of a third toxin, *C. difficile* transferase (CDT) (Gerding et al. 2014). Recently, Wang et al. (2015) have demonstrated the lethality of this third toxin and have shown that vaccination provides protection in rodent models.

CDT is a binary toxin comprised of two independently non-toxic components, the cell-binding/translocation component, CDTb, and the enzymatic ADP-ribosyltransferase (ART) component, CDTa. Proteolytically-activated CDTb binds lipolysis-stimulated lipoprotein receptors (LSRs) and forms heptameric complexes on the host cell surface. There, it recruits CDTa and the intact binary toxin is transported into the cell. Endosomal acidification triggers CDTb-mediated pore formation and translocation of CDTa to the cytoplasm. There, CDTa binds intracellular NAD⁺ and transfers the ADP-ribose moiety to monomeric actin, destabilizing the actin cytoskeleton. Disruption of cortical actin leads to the formation of extracellular microtubule protrusions that promote further bacterial adhesion and colonization (Gerding et al. 2014).

Although CDTa is primarily categorized as the enzymatic component of binary toxin, it is also required to participate in coordination of complex formation with CDTb. The protein accomplishes its dualistic functions by splitting the responsibility between structurally similar but mechanistically unique domains, the ~26 kDa N-terminal CDTb-interacting domain (CDTaBID; residues 1–215) and the ~23 kDa C-terminal catalytic ART domain (residues 216–420). We can take advantage of this arrangement to express and characterize the structure-function relationships of individual CDTa domains in isolation by solution NMR. To that end, we have recently reported assignments of the enzymatic C-terminal domain (^{216–420}CDTa) (Roth et al. 2016) and here present NMR assignments for the *Clostridium difficile* CDTb-interacting domain of CDTa, CDTa-BID.

Methods and experiments

Sample Preparation

We have generated a 222 amino acid (~26 kDa) truncated version of the *Clostridium difficile* binary toxin component, CDTa. The purified protein sequence consists of a single non-native methionine residue followed by the first 221 residues of mature CDTa. It also harbors a functionally silent cysteine-to-alanine mutation at position two (C2A) that has been shown to facilitate protein purification and stability (Xie et al. 2014). This construct represents the CDTb-interacting domain (CDTaBID) and is hereafter termed 1mBID. The 1mBID fragment was cloned in frame with an N-terminal His₆-SUMO fusion partner to enhance expression and purification of soluble protein and transformed into *Escherichia coli* BL21(DE3) cells. For NMR studies, [¹H, ¹⁵N]-1mBID was prepared by isopropyl-β-D-thiogalactopyranoside (IPTG)-induced expression of His₆-SUMO-1mBID overnight at 20 °C in M9 minimal media containing ¹⁵NH₄Cl as the sole nitrogen source. For deuterated, doubly-labeled samples, cells were adapted to growth in 99.9% D₂O by successive passage of cells into [¹⁵N, ¹³C]-enriched media containing 90%, 95%, 99%, and 99.9% D₂O. Cells were grown overnight between each passage at 37 °C in M9 minimal media containing ¹⁵NH₄Cl and protonated ¹³C-glucose as the sole nitrogen and carbon sources, respectively. Protein expression was induced with 0.3 mM IPTG at 20 °C overnight. Cells were pelleted by centrifugation and resuspended in lysis buffer containing 20 mM Tris pH 7.4, 500 mM NaCl, and 5 mM Imidazole. Cells were lysed by sonication, the soluble lysate was applied to a 5 mL Ni²⁺-charged IMAC FF affinity column (G.E. Healthcare), and the His₆-SUMO-1mBID was eluted in the same buffer containing 500 mM Imidazole. The His₆-SUMO tag was removed by overnight incubation with Ulp1 protease while simultaneously buffer exchanged to lysis buffer. The His₆-SUMO cleaved protein was applied to a 5 mL HisTrap HP column and the flow-through was collected, containing purified 1mBID. The purified protein was dialyzed against NMR buffer and concentrated by centrifugal filtration. A typical sample contained 0.4 mM 1mBID in 15 mM MES pH 6.0, 10 mM NaCl, 5 mM DTT, 0.05 mM EDTA, 3 mM NaN₃ and 10% D₂O.

NMR experiments

All NMR experiments were acquired at 298 K on a Bruker Avance III 950 MHz spectrometer equipped with a z-gradient cryogenic probe. A 2D [¹H-¹⁵N]-TROSY-HSQC, shown in Fig. 1, was used as the root spectrum to assign backbone resonances via pairwise comparison of inter- and intra-residue ¹³C_α, ¹³C_β and ¹³C' chemical shifts. To overcome signal-to-noise limitations observed with fully-protonated samples, we expressed and purified deuterated [¹⁵N, ¹³C]-1mBID and exploited TROSY-based pulse sequences to enhance sensitivity and resolution. Triple resonance HNCACB, HN(CO)CACB, HNCA, HN(CO)CA, HNCO, and HN(CA)CO experiments were collected on ~0.4 mM [²H, ¹⁵N, ¹³C]-labeled 1mBID samples back-exchanged in 90% H₂O/10% D₂O NMR buffer (15 mM MES pH 6.0, 10 mM NaCl, 5 mM DTT, 50 μM EDTA, and 3 mM NaN₃) at 25 °C. All 3D datasets were acquired by non-uniform sampling (NUS) of 10% of the linear points using a sine-weighted Poisson-gap scheduler for the indirect dimensions (Hyberts et al. 2012). Reconstruction of sparse data was achieved with NESTA-NMRv1.0 using the default settings for L1 regularization (Sun et al. 2015). Talos+ was used to determine secondary

structure probabilities based on experimentally derived H^N , N , Ca , $C\beta$ and C' chemical shifts (Fig. 2). ^{15}N - $\{^1H\}$ heteronuclear NOE experiments were collected at 950 MHz by fully interleaving NOE and reference spectra. A series of experiments was acquired with relaxation delays of 3-s, 4-s, and 5-s to ensure the steady state NOE had been achieved. Fig. 2 shows NOE measurements derived from an experiment utilizing a 5-s delay. NMR data were processed with NMRPipe (Delaglio et al. 1995) and analyzed with CcpNmr Analysis (Vranken et al. 2005). All proton chemical shifts were referenced to external trimethylsilyl propanoic acid (TSP) at 25 °C (0.00 ppm) with respect to residual H_2O (4.698 ppm). 1H - ^{15}N and 1H - ^{13}C chemical shifts were indirectly referenced using zero-point frequency ratios of 0.101329118 and 0.251449530, respectively.

Assignments and data deposition

Backbone assignments were obtained for the CDTb-interacting domain (BID) of the *Clostridium difficile* binary toxin enzymatic component, CDTa. The 222 amino acid N-terminal construct described here (1mBID) features a non-native methionine followed by the first 221 residues of mature CDTa (lacking signal peptide) and includes a functionally silent cysteine-to-alanine mutation at position two. The well-dispersed 2D $[^1H$ - $^{15}N]$ -TROSY-HSQC spectrum of 1mBID is shown in Fig. 1. Under conditions used in this experiment, 100% (201/201) of observable 1H - ^{15}N correlations were assigned unambiguously. At the selected contour level, peaks corresponding to S75 and G189 fell below the observable threshold. Consequently, the coordinates of these two residues were each denoted with an asterisk (*). In total, 843 of 873 (97 %) backbone resonances were assigned, including 94 % of $^1H^N$ protons and ^{15}N amides (201/213), 98 % of Ca (218/222), 97 % of $C\beta$ (210/216) and 96 % of C' (214/222) resonances. Residues in dynamic regions that were elusive in the crystal structure, 2WN4 (the proximate 27 amino acids and the $\beta 4$ - $\beta 5$ loop), were readily observed. Twelve residues (M-1, V1, I10, E11, E27, D68, R76, R114, K153, G154, Q188 and D203) were missing or severely broadened in the ^{15}N -edited 2D-HSQC, likely due to solvent exchange or conformational averaging on an intermediate timescale. Of the twelve, four residues located in the unstructured N-terminal region (M-1, I10, and E11) and the $\alpha 4$ - $\beta 5$ loop (K153) failed to provide heteronuclear Ca , $C\beta$, and C' assignments as well. The chemical shift assignments from these experiments were all deposited in the BioMagResBank (www.bmrb.wisc.edu) under accession number 26044.

The 1mBID assignments determined here were used to generate a chemical shift index and map secondary structure. As shown in Fig. 2, the predicted secondary structure of the CDTb-interacting domain of CDTa, 1mBID, comprises four alpha-helices and five beta-strands ($\alpha 1$:residues 25–35, $\alpha 2$:residues 40–65, $\alpha 3$:residues 75–89, $\alpha 4$:residues 123–135, $\beta 1$:residues 97–99, $\beta 2$:residues 161–166, $\beta 3$:residues 184–186, $\beta 4$:residues 197–201 and $\beta 5$:residues 206–214). Analysis of heteronuclear NOEs reveals dynamic regions of the protein sequence and is in good agreement with the secondary structure model predicted by Ca , $C\beta$, and C' chemical shifts. Moreover, the 1mBID secondary structure closely resembles that of the CDTa crystal structure, 2WN4, with minor differences owing to secondary structure boundaries or regions in which the selection criteria for secondary structure falls just below the confidence threshold (see residues 68–71 and 138–142). Such consistency in secondary structure between the 1mBID and full-length CDTa warrants the

use of this N-terminal construct for NMR-based characterization including CDTa-CDTb interaction mapping as well as screening of small molecule inhibitors of binary toxin complex formation.

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References

- Delaglio F, Grzesiek S, Vuister GW, et al. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995; 6:277–293. [PubMed: 8520220]
- Gerding DN, Johnson S, Rupnik M, Aktories K. Clostridium difficile binary toxin CDT: mechanism, epidemiology, and potential clinical importance. *Gut Microbes*. 2014; 5:15–27. [PubMed: 24253566]
- Hyberts SG, Arthanari H, Wagner G. Applications of non-uniform sampling and processing. *Top Curr Chem*. 2012; 316:125–148. [PubMed: 21796515]
- Lessa FC, Mu Y, Bamberg WM, et al. Burden of Clostridium difficile infection in the United States. *N Engl J Med*. 2015; 372:825–834. [PubMed: 25714160]
- Perelle S, Gibert M, Bourlioux P, et al. Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by Clostridium difficile CD196. *Infect Immun*. 1997; 65:1402–1407. [PubMed: 9119480]
- Roth BM, Godoy-Ruiz R, Varney KM, et al. (1)H, (13)C, and (15)N resonance assignments of an enzymatically active domain from the catalytic component (CDTa, residues 216–420) of a binary toxin from Clostridium difficile. *Biomol NMR Assign*. 2016
- Sun S, Gill M, Li Y, et al. Efficient and generalized processing of multidimensional NUS NMR data: The NESTA algorithm and comparison of regularization terms. *J Biomol NMR*. 2015; 62:105–117. [PubMed: 25808220]
- Vranken WF, Boucher W, Stevens TJ, et al. The CCPN data model for NMR spectroscopy: development of a software pipeline. *Proteins*. 2005; 59:687–696. [PubMed: 15815974]
- Wang S, Rustandi RR, Lancaster C, et al. Toxicity assessment of Clostridium difficile toxins in rodent models and protection of vaccination. *Vaccine*. 2015; 34:1319–1323. [PubMed: 26614590]
- Xie J, Horton M, Zorman J, et al. Development and optimization of a high-throughput assay to measure neutralizing antibodies against clostridium difficile binary toxin. *Clin Vaccine Immunol*. 2014; 21:689–697. [PubMed: 24623624]

Figure 2.

Characterization of the N-terminal CDTb-interaction domain of CDTa (1mBID) from *Clostridium difficile* based on NMR chemical shifts. (a) Raw chemical shift deviations of $C\alpha$ and $C\beta$ carbons ($\delta(C\alpha) - \delta(C\beta)$) with respect to corresponding random coil values are plotted against residue number. Positive and negative values indicate α -helix and β -strand character, respectively. Panel (b) shows the probability of secondary structure formation as predicted by Talos+, with α -helices represented by blue bars and β -strands by red bars. Amino acid stretches that meet the requirements for secondary structure definition are indicated by blue and red boxes as $\alpha 1$ – $\alpha 4$ and $\beta 1$ – $\beta 5$. Heteronuclear NOE relaxation parameters acquired at 950 MHz with a 5-s relaxation delay (closed black circles) indicate mobile regions of 1mBID that are consistent with Talos+ predictions.