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¹H, ¹³C, and ¹⁵N Resonance Assignments of an Enzymatically Active Domain from the Catalytic Component (CDTa, residues 216-420) of a Binary Toxin from *Clostridium difficile*

Braden M. Roth^{*}, Raquel Ruiz^{*}, Kristen M. Varney^{*}, Richard R. Rustandi[†], and David J. Weber[‡]

David J. Weber: dweber@umaryland.edu

^{*}Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 108 N. Greene St., Baltimore, Maryland 21201

[†]Department of Vaccine Analytical Development, Merck Research Laboratories, 770 Sumneytown Pike, West Point, PA 19486

[‡]Dept. of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 108 N. Greene St., Baltimore, MD 21201, Telephone: (410) 706-4354, Fax: (410) 706-0458

Abstract

Clostridium difficile is a bacterial pathogen and is the most commonly reported source of nosocomial infection in industrialized nations. Symptoms of *Clostridium difficile* infection (CDI) include antibiotic-associated diarrhea, pseudomembranous colitis, sepsis and death. Over the last decade, rates and severity of hospital infections in North America and Europe have increased dramatically and correlate with the emergence of a hypervirulent strain of *C. difficile* characterized by the presence of a binary toxin, CDT (*Clostridium difficile* toxin). The binary toxin consists of an enzymatic component (CDTa) and a cellular binding component (CDTb) that together form the active binary toxin complex. CDTa harbors a pair of structurally similar but functionally distinct domains, an N-terminal domain (residues 1-215; ¹⁻²¹⁵CDTa) that interacts with CDTb and a C-terminal domain (residues 216-420; ²¹⁶⁻⁴²⁰CDTa) that harbors the intact ADP-ribosyltransferase (ART) active site. Reported here are the ¹H, ¹³C, and ¹⁵N backbone resonance assignments of the 23 kDa, 205 amino acid C-terminal enzymatic domain of CDTa, termed ²¹⁶⁻⁴²⁰CDTa. These NMR resonance assignments for ²¹⁶⁻⁴²⁰CDTa represent the first for a family of ART binary toxins and provide the framework for detailed characterization of the solution-state protein structure determination, dynamic studies of this domain, as well as for NMR-based drug discovery efforts.

Keywords

Clostridium difficile; CDI; CDTa; Binary toxin; ADP-ribosyltransferase

Correspondence to: David J. Weber, dweber@umaryland.edu.

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

Biological context

Clostridium difficile is a spore-forming, gram-positive bacterium and causative agent of *C. difficile* infection (CDI). Infection is commonly associated with disruption of the gut flora by antibiotic treatment and results in symptoms ranging from watery diarrhea to pseudomembranous colitis (PMC) or death. Over the last decade, rates and severity of hospital infections in North America and Europe have increased dramatically. *C. difficile* is now the most commonly reported nosocomial pathogen, accounting for more than 12% of all health care facility infections and 15–25% of antibiotic-associated diarrhea (AAD) episodes (Gerding and Lessa 2015). This increase coincides with the emergence of a hypervirulent strain of *C. difficile*, NAP1 (Perelle et al. 1997).

Historically, non-epidemic strains of *C. difficile* produce two large enterotoxins, TcdA and TcdB, which inhibit signaling pathways by glucosylating small GTPases. In addition to these large toxins, the NAP1 epidemic strain encodes other virulence factors, most notably a third toxin, termed the *Clostridium difficile* binary toxin (CDT). Like other members of a diverse family of bacterial toxins, CDT is an ADP-ribosyltransferase (ART) that kills host cells through covalent modification of essential regulators of cellular function. As the name suggests, the binary toxin is produced as a pair of individually non-toxic proteins (CDTa and CDTb), which interact to form the active CDT binary toxin complex. Proteolytically activated heptamers of CDTb, the cell-binding component, recognize cell surface lipolysis-stimulated lipoprotein receptors (LSR) and deliver the enzymatic component, CDTa, into the cell (Papatheodorou et al. 2011). Following endocytosis of the CDT/LSR complex, CDTb also forms a pore in the endosomal membrane as necessary to translocate CDTa into the cytoplasm. There, CDTa binds intracellular NAD⁺ and transfers its ADP-ribose moiety to actin monomers, destabilizing the actin cytoskeleton and promoting the formation of extracellular microtubule protrusions that enhance further bacterial colonization.

Crystal structures reveal that CDTa is secreted as a 48 kDa protein possessing a pair of similarly structured but mechanistically distinct domains (Sundriyal et al. 2009); the N-terminal domain (residues 1–215; ^{1–215}CDTa) mediates CDTb interaction while the C-terminal domain (residues 216–420; ^{216–420}CDTa) harbors ART activity (Gülke et al. 2001). Because they function independently, the domains were expressed here separately for more rapid characterization by NMR spectroscopy. NMR provides a unique opportunity to probe the structural and dynamic features of CDTa that contribute to substrate binding and toxin complex formation. To that end, we present the first NMR assignments of the enzymatic C-terminal domain of CDTa, ^{216–420}CDTa.

Methods and experiments

Sample Preparation

A truncated version of *Clostridium difficile* binary toxin component, CDTa, was engineered to span the C-terminal 205 amino acid ADP-ribosyltransferase domain (residues 216–420; termed ^{216–420}CDTa). The ^{216–420}CDTa construct was prepared 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. A single colony was used to inoculate

M9 minimal media containing $^{13}\text{C}_6$ -glucose and $^{15}\text{NH}_4\text{Cl}$ as the sole carbon and nitrogen sources, respectively. Expression of His₆-SUMO-²¹⁶⁻⁴²⁰CDTa was induced with 0.3 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 25 °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 Ni⁺-charged IMAC FF affinity column (G.E. Healthcare), and the His₆-SUMO-²¹⁶⁻⁴²⁰CDTa 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 cleaved protein was applied to a 5 mL HisTrap HP column and the flow-through was collected, containing purified ²¹⁶⁻⁴²⁰CDTa. The pure enzymatic domain was dialyzed against NMR buffer and concentrated. A typical sample contained 0.4 mM ²¹⁶⁻⁴²⁰CDTa in 15 mM HEPES pH 7.0, 50 mM NaCl, 5 mM DTT, 0.05 mM EDTA, and 0.02% NaN₃ and 10% D₂O.

NMR experiments

All NMR experiments were recorded at 298 K on Bruker Avance 800 and Avance III 950 MHz spectrometers equipped with z-gradient cryogenic probes. Backbone $^1\text{H}^{\text{N}}$, ^{15}N , $^{13}\text{C}'$, $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ resonances were assigned by pairwise comparison of intra- and inter-residue chemical shifts acquired in complementary 3D HNCACB, CBCA(CO)NH, HNCA, HN(CO)CA, HNCO, HN(CA)CO, and 2D [^1H - ^{15}N]-TROSY-HSQC spectra. Additional sidechain resonances were obtained using CC(CO)NH spectra with 12 ms and 18 ms TOCSY mixing times. Assignments were confirmed by through-space 3D ^{15}N -NOESY-HSQC and ^{15}N -edited HSQC-NOESY-HMQC experiments. ^{15}N - $\{^1\text{H}\}$ heteronuclear NOE experiments were measured at 800 MHz by fully interleaving NOE and reference spectra as previously described (Farrow et al. 1994) and were acquired as a series of three experiments with relaxation delays of 3-s, 4-s, and 5-s to ensure the steady state NOE had been achieved. Data were processed using NMRPipe (Delaglio et al. 1995) and analyzed with CCPN (Vranken et al. 2005). ^1H chemical shifts were referenced to internal trimethylsilyl propanoic acid (TSP) while ^{15}N and ^{13}C chemical shifts were indirectly calibrated. Talos+ (Shen et al. 2009) was used to generate secondary structure and torsion angle (ϕ, ψ) predictions based on experimentally derived H^{N} , N, C', C α and C β chemical shifts.

Assignments and data deposition

Assignment of the C-terminal enzymatic domain of CDTa, described here, represents the first set of NMR resonance assignments for a family of related binary toxins produced by *Clostridium* or *Bacillus* species. The well-dispersed 2D [^1H - ^{15}N]-TROSY-HSQC spectrum of ²¹⁶⁻⁴²⁰CDTa is shown in Fig. 1. Under conditions used in these experiments, 99% (173/175) of the observable backbone ^1H - ^{15}N correlations were assigned unambiguously. Two weak correlations, each marked with an asterisk in Fig. 1, were found to be located in the unstructured $\beta 3$ - $\beta 4$ region based on their amino acid type, but inter-residue connectivity required for unambiguous assignment was lacking for these two correlations. Other residues having missing or severely broadened correlations in the ^{15}N -edited 2D-HSQC was the result of solvent exchange, conformational averaging, and/or fast timescale motions as identified for residues in the ADP-ribosyltransferase turn-turn (ARTT) loop (residues 379-387) via the ^{15}N - $\{^1\text{H}\}$ heteronuclear NOE experiment (Fig. 2e). When mapped onto the

crystal structure of substrate-bound CDTa (2WN6) (Sundriyal et al. 2009), residues with fast and/or slow timescale motions were located within the active site or at the N-terminus of this construct. These include ^1H - ^{15}N correlations for residues S216, S217, V224 in the N-terminal region and S304-K306, S347, S350-A357, R359, K360, L363, Y375, and G384 at or near the active site. Conformational averaging and fast timescale motion within the enzyme's active site is consistent with a strain alleviation mechanism that is described for *Clostridium* ADP-ribosylation, in which flexibility of the active site is essential for substrate binding and ribosyltransferase activity (Sundriyal et al. 2009; Tsurumura et al. 2013). Although conformational dynamics reduced the number of observable correlations, over 93% (727/806) of all backbone resonances were assigned including 94% of C α (193/205) and 92% of C (188/205) resonances. In addition, 46% (178/385) of all ^{13}C sidechain assignments were completed including 92% (175/190) of the C β assignments. The chemical shift assignments from these experiments were all deposited in the BioMagResBank (www.bmrb.wisc.edu) under accession number 25665.

The backbone C α , C β and C' assignments determined here were used next to generate a chemical shift index, predict bond angles, and map the secondary structure of $^{216-420}\text{CDTa}$. As shown in Fig. 2c, the resulting secondary structure closely resembles that of the apo-CDTa crystal structure, 2WN4, (Sundriyal et al. 2009) with minor differences in individual secondary structure borders. The predicted secondary structure of the enzymatic domain of *C. difficile*, $^{216-420}\text{CDTa}$, in solution includes five alpha-helices and seven beta-strands (α 1: residues 224-235, α 2: residues 245-254, α 3: residues 258-266, α 4: residues 277-290, α 5: residues 323-333, β 1: residues 297-303, β 2: residues 337-339, β 3: residues 347-349, β 4: residues 361-367, β 5: residues 385-390, β 6: residues 395-406 and β 7: residues 410-419). Such consistency in the secondary structure determination and the identification of dynamic regions of the enzyme within its active site, as predicted, warrants the use of this C-terminal construct for NMR-based characterization including screening of small molecule inhibitors that target the active site of CDTa.

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Fig. 1. 2D [^1H - ^{15}N]-TROSY-HSQC of the ADP-ribosyltransferase domain of CDTa ($^{216-420}\text{CDTa}$) recorded on a Bruker Avance III 950 MHz spectrometer at pH 7.0 and 298 K. An enlarged view of the most crowded region of the spectrum is shown in the top-left corner. Residue type and number indicate assignments from backbone amide N-H correlations. The prime (') notation represents correlations in the N-terminus (residues 218-234) arising from a minor secondary conformation. The two weak correlations marked with an asterisk are located in the unstructured $\beta 3$ - $\beta 4$ region and lack inter-residue connectivity required for unambiguous assignment.

Fig. 2. Characterization of the C-terminal enzymatic domain of CDTa ($^{216-420}\text{CDTa}$) from *Clostridium difficile* based on NMR chemical shifts. **(a)** Raw chemical shift deviations of $\text{C}\alpha$ and $\text{C}\beta$ carbons ($\delta(\text{C}\alpha) - \delta(\text{C}\beta)$) with respect to corresponding random coil values are plotted against residue number. Positive and negative values indicate α -helix and β -strand character, respectively. **(b)** The probability of secondary structure formation as predicted by Talos+. **(c)** Comparison of predicted NMR-based secondary structure elements with those of the crystal structure, 2WN4 (Sundriyal et al. 2009). In **b** and **c**, α -helices are represented as blue bars, β -strands as red bars, and the Random Coil Index as closed black circles. **(d)** Talos+ predicted phi and psi angles are represented by white boxes and black diamonds, respectively. **(e)** Heteronuclear NOE backbone relaxation parameters acquired at 800 MHz is plotted against residue number. White diamonds represent the average of three experiments collected with relaxation delays of 3, 4, and 5 seconds. Shading in panel **e** corresponds to the ADP-ribosyltransferase turn-turn (ARTT) loop (residues 379-387). Error bars in panels **d** and **e** represent one standard deviation above and below the mean.