**Clostridium difficile** Toxins A and B Are Cation-dependent UDP-glucose Hydrolases with Differing Catalytic Activities*  

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William P. Ciesla, Jr.‡ and David A. Bobak¶§

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¶To whom correspondence should be addressed: Box 485-HSC, University of Virginia School of Medicine, Charlottesville, Virginia 22908. Tel.: 804-924-9673; Fax: 804-977-5323; E-mail: dab2g@virginia.edu.

§The abbreviations used are: UDP-glucose, uridine diphosphate-glucose; hydrolyase, UDP-glucosylhydrolase; DTT, dithiothreitol; TEA, triethanolamine.

Toxins A and B of **Clostridium difficile** are UDP-glucose glucosyltransferases that exert their cellular toxicity primarily through their abilities to monoglucosylate, and thereby inactivate, Rho family small GTPases. Toxin A also hydrolyzes UDP-glucose, although this activity is not well characterized. In this study, we measured the kinetics of UDP-glucose hydrolysis by toxins A and B and found significant differences in the catalytic activities of these two structurally homologous toxins. The toxins displayed similar Michaelis constants (**K**<sub>m</sub>) for UDP-glucose, but the maximal velocity (**V**<sub>max</sub>) of toxin B was ~5-fold greater than that of toxin A. Toxins A and B exert their enzymatic actions intracellularly, and, interestingly, we found that each toxin absolutely required **K**<sup>+</sup> for optimal hydrolyase activity; **Na**<sup>+</sup> was inactive. The toxins also required certain divalent cations for activity and exhibited a significantly greater **V**<sub>max</sub> and lower **K**<sub>m</sub> in the presence of Mn<sup>2+</sup> as compared with Mg<sup>2+</sup>. We conclude that C. difficile toxins A and B are cation-dependent UDP-glucose hydrolases that differ significantly in their catalytic activities, a finding that may have important implications in understanding their different cytotoxic effects.

**Clostridium difficile** is the leading cause of nosocomial diarrhea in the United States and causes disease through the actions of its two exotoxins (toxin A and toxin B) (1–3). They are the largest monomeric protein toxins currently known; toxin A contains 2710 amino acid residues (M<sub>r</sub> of 308,000), and toxin B has 2366 residues (M<sub>r</sub> of 270,000). In animals, the administration of toxin A causes fluid secretion into the intestinal lumen, epithelial cell disruption, and an intense inflammatory reaction. The exact way that the toxins cause disease is incompletely understood, but it was recently discovered that the major intracellular targets of toxins A and B are the family of signal-transducing small G-proteins known as Rho GTPases (4, 5). Rho proteins regulate a variety of cellular functions, including cytoskeletal organization, cell growth and differentiation, apoptosis, and nuclear signaling (6–8). Using cellular uridine diphosphate-glucose (UDP-glucose)<sup>1</sup> as the glucose donor, the toxins monoglucosylate members of the Rho family of GTPases (Rho, Rac, and Cdc42). This monoglucosylation disrupts the normal function of the Rho GTPases and causes a variety of effects on intoxicated cells including dysregulation of the actin cytoskeleton, cell rounding, cytotoxicity, and altered cellular signaling (9, 10).

Although toxin A and toxin B are structurally homologous glucosyltransferases, significant differences exist between their **in vivo** and **in vitro** effects. Each is cytotoxic to cultured cells, but for most cell lines, toxin B is 100–1000 times more potent than toxin A (3). Only toxin A causes intestinal pathology in animal models of disease; toxin B has no discernible effects (11). The reasons for these differences are unclear. The two toxins may have different rates of cellular binding and uptake, or they may differ in their abilities to glucosylate their specific target proteins, i.e. the Rho family GTPases. Because the specific cell surface toxin receptors have not been identified for human cells, detailed information on toxin binding and uptake is not available. Similarly, the lack of a quantitative assay amenable to kinetic studies has prevented detailed biochemical study of the toxins.

Interestingly, the monoglucosylation of Rho GTPases is not the only enzymatic activity described for toxin A. In the absence of an acceptor protein, toxin A can hydrolyze UDP-glucose to UDP and glucose (5). The hydrolyase activity of toxin A is similar to that of certain microbial ADP-ribosyltransferases, such as cholera toxin, that ADP-ribosylate target proteins but also hydrolyze NAD to nicotinamide and ADP-ribose in the absence of an acceptor protein (12, 13). As with the glucosyltransferase activity, the hydrolyase activities of the C. difficile toxins have not been studied in detail. For example, the characteristics and kinetics of the toxin-mediated hydrolyase reactions are not known.

In this study, we found that the hydrolyase activity (**V**<sub>max</sub>) of toxin B was approximately 5 times greater than that of toxin A, whereas the **K**<sub>m</sub> for UDP-glucose was similar for both toxins. In addition, we found that the toxins required potassium for hydrolyase activity and that potassium activated toxin function by increasing toxin **V**<sub>max</sub> with little effect on the **K**<sub>m</sub> for UDP-glucose. Sodium was inactive in supporting hydrolyase activity. The toxins also required manganese or magnesium for optimal hydrolyase activity, with manganese being the more potent activator. This report provides the first quantitative analysis of the kinetics of any enzymatic activity of the C. difficile toxins, characterizes the cation dependence of the toxins, and demonstrates that the hydrolyase activity of toxin B is significantly greater than toxin A.

**EXPERIMENTAL PROCEDURES**

**Materials**—Purified C. difficile toxins A and B were a gift from D. Lyerly (TechLab, Inc., Blacksburg, VA). UDP-glucose was from Boehringer Mannheim. [14C]UDP-glucose (287.4 mCi/mmol) and [14C]Glucose (14.6 Ci/mmol) were from NEN Life Science Products. AG1-X2 anion-exchange resin was from Bio-Rad. All other chemicals and reagents were from commercial sources and were analytical or...
highest available grade.

UDP-glucose Hydrolyase Assay—The initial assay conditions were similar to those reported by Just et al. for their TLC-based assay of toxin-mediated UDP-glucose hydrolyase activity (5). Our column-based assay was modified from a similar assay originally developed by Moss and Vaughan (12). A 120-µl sample containing 50 mM triethanolamine (TEA, pH 7.8), 150 mM KC1, 1 mM dithiothreitol (DTT), 2 mM MgCl₂, 100 µM UDP-glucose (~3800 dpm/assay), and with and without toxin (100 µg/ml toxin A or 88 µg/ml toxin B, representing equimolar toxin concentrations) was incubated at 37 °C for the time indicated. Following incubation, duplicate 50-µl samples of the reaction mixture were added to separate 1.0-ml columns of the AG1-X2 resin, which were then flushed four times with 1 ml of water. The eluates, containing [¹⁴C]glucose, were collected, and the radioactivity was measured in a liquid scintillation counter. For each condition, control assays without toxin were included and, if present, the radioactivity in these control eluates was subtracted from the appropriate toxin-containing assay to calculate toxin-specific hydrolyase activity. Other experiments contained variations of these conditions and/or substitution of various reaction times, buffers, salts, cations, etc., and are described in the text and figure legends. The assays for the kinetic experiments were performed as above, using the specific reaction conditions as stated in the respective figure legends. In each experiment, the ratio of [¹⁴C]-labeled to unlabeled UDP-glucose was held constant.

Data Analysis and Presentation—The data shown in each figure, or referred to in the text as “not shown,” represent the results of at least three separate experiments and each experiment used duplicate assays per condition. The data are presented as the mean ± the standard deviation of the pooled experiments. Plots and curve fitting were performed using the Kaleidograph graphics application program (Abelbeck software). The kinetic parameters, K_ₑ (Michaelis constant) and V_ₑ₅ₐₓ (maximal enzyme velocity), were obtained from the pooled data of several experiments using the solver function of the Microsoft Excel (version 5.0) computer program. This approach uses a nonlinear least squares analysis to obtain a best fit between the velocity/substrate curve from the experimental data and the curve generated using the Michaelis-Menten equation.

RESULTS

UDP-glucose hydrolyase activity for C. difficile toxin A was initially described by Just and colleagues (5). We wished to investigate the hydrolyase activity of the toxins in greater detail, however, and needed to devise a hydrolyase assay that was more quantitative and convenient than the TLC-based assay used in that paper. We therefore adapted a column-based assay that was originally developed to measure the hydrolyase activity of NAD transferase enzymes such as choler toxin (12). The assay is based on the fact that [glucose-¹⁴C]UDP-glucose is negatively charged and binds to AG1-X2 anion exchange resin. The free UDP generated by hydrolysis also binds to the resin. The product of the hydrolysis reaction, however, is [¹⁴C]glucose, an uncharged compound that passes through the column and serves as a measure of the amount of free glucose generated by the hydrolysis reaction. Using the initial assay conditions outlined under “Experimental Procedures,” we found that >99.5% of applied [glucose-¹⁴C]UDP-glucose was retained by the column and approximately 99% of applied [¹⁴C]glucose passed freely through the column (data not shown). These results established the validity of the previously described NAD hydrolyase assay for use as a measure of UDP-glucose hydrolyase activity.

We first investigated the time course of UDP-glucose hydrolysis for toxins A and B over a range of substrate concentrations (Fig. 1, A and B). As expected, toxin B did indeed display UDP-glucose hydrolyase activity; however, we additionally found that the hydrolyase activity of toxin B was much greater than that of toxin A. To characterize the differences in catalytic activities between the two toxins in more detail, we next determined the kinetic parameters of each toxin’s UDP-glucose hydrolyase activity. The effects of varied substrate concentrations on the hydrolyase activities of toxins A and B are seen in the velocity/substrate curves for each toxin (Fig. 2, A and B). These data displayed saturation kinetics when the enzyme activity (mol of UDP-glucose hydrolyzed/mol of toxin⁻¹·h⁻¹) was plotted as a function of substrate concentration. For the data shown in Fig. 2, the Michaelis constant (Kₘ) for UDP-glucose was 142.3 µM for toxin A and 175.5 µM for toxin B. Consistent with the results seen in the time-course experiment, we found the hydrolyase activity of toxin B to be significantly greater than that of toxin A. For toxin A, the maximal enzyme velocity (V_ₑ₅ₐₓ) was 17.3 (mol of UDP-glucose hydrolyzed/mol of toxin⁻¹·h⁻¹) as compared with a value of 89.8 (mol of UDP-glucose hydrolyzed/mol of toxin⁻¹·h⁻¹) for toxin B.

Next, we sought to determine how other factors could influence the UDP-glucose hydrolyase activity of the toxins. The optimal temperature of toxin-mediated hydrolysis was between 37 and 40 °C, and that minimal hydrolyase activity occurred at 4 °C or at 55 °C (data not shown). In addition, the toxins were most active in the pH range of 7.8–8.0, had diminished activity...
at more acidic conditions, and were inactive at a pH below 5.0 (data not shown). The addition of DTT at 1 or 10 mM had little or no effect on the hydrolase activity of either toxin (data not shown). This finding was somewhat surprising because of previously published results indicating that the conserved cysteine residues of the toxin B were important in cytotoxicity caused by the toxin (14). Finally, low concentrations (0.1%) of nonionic detergents such as Nonidet P-40 or Tween-20 had no measurable effect on the activities of the two toxins, whereas similar concentrations of sodium cholate, an ionic detergent, inactivated toxin-mediated hydrolysis (data not shown).

Because the toxins are known to exert their enzymatic actions intracellularly, we next examined the effects of potassium on toxin-mediated hydrolytic activity. As outlined in Fig. 3 (A and B), we found that the toxins absolutely required potassium for optimal activity and we further noted a clear concentration-dependent enhancement of UDP-glucose hydrolysis by potassium. Equivalent concentrations of NaCl, however, did not support toxin activity. These data suggest, then, that the activating effects of potassium were not simply a result of changes in ionic strength, but are relatively specific for the effects of the potassium ion itself. Intriguingly, the normal intracellular concentration of ~150 mM is near the EC50 of KCl for toxin-mediated hydrolase activity.

Because we found that potassium was required for toxin-mediated UDP-glucose hydrolysis, we next studied how potassium would affect the kinetic parameters of this activity for each toxin. Using a method analogous to that used for the experiments shown in Fig. 2, we generated velocity/substrate curves for toxins A and B as a function of the concentration of KCl. As seen in Fig. 4, increasing the concentration of KCl markedly increased the Vmax (mol of UDP-glucose hydrolyzed/mol of toxin h−1) for toxin A: 50 mM KCl = 7.65, 150 mM KCl = 17.3, and 500 mM KCl = 34.8. For toxin A, the Km for UDP-glucose (expressed in µM), on the other hand, exhibited minimal change: 50 mM KCl = 143.5, 150 mM KCl = 142.3, 500 mM KCl = 163.8. In the case of toxin B, we also observed a KCl-dependent increase in the Vmax (mol of UDP-glucose hydrolyzed/mol of toxin h−1): 50 mM KCl = 38.5, 150 mM KCl = 89.7, 500 mM KCl = 163.2 (Fig. 5). For toxin B, the Km for UDP-glucose (expressed in µM) was also little changed by increased KCl concentrations: 50 mM KCl = 157.4, 150 mM KCl = 175.5, 500 mM = 173.5.

![Fig. 2. Effect of substrate concentration on the UDP-glucose hydrolase activities of toxins A and B.](image1)

![Fig. 3. Potassium activates the UDP-glucose hydrolase activities of toxins A and B.](image2)
concentration varied from 15.62 to 2000 mM as indicated), and toxin A (100 μg/ml); reactions were incubated at 37 °C for 1 h. Each value represents the mean ± S.D. of at least three experiments.

FIG. 4. Effect of potassium concentration on the kinetics of the UDP-glucose hydrolase activity of toxin A. The hydrolase assay was performed as described under “Experimental Procedures.” The buffer contained KCl (concentration was 50, 150, or 500 mM as indicated), 2 mM MgCl₂, 50 mM TEA (pH 7.8), 1 mM DTT, UDP-glucose (concentration varied from 15.62 to 2000 μM as indicated), and toxin A (100 μg/ml); reactions were incubated at 37 °C for 1 h. Each value represents the mean ± S.D. of at least three experiments.

FIG. 5. Effect of potassium concentration on the kinetics of the UDP-glucose hydrolase activity of toxin B. The hydrolase assay was performed as described under “Experimental Procedures.” The buffer contained KCl (concentration was 50, 150, or 500 mM as indicated), 2 mM MgCl₂, 50 mM TEA (pH 7.8), 1 mM DTT, UDP-glucose (concentration varied from 15.62 to 2000 μM as indicated), and toxin A (100 μg/ml); reactions were incubated at 37 °C for 1 h. Each value represents the mean ± S.D. of at least three experiments.

FIG. 6. The UDP-glucose hydrolase activities of toxins A and B require divalent cations. The hydrolase assay was performed as described under “Experimental Procedures.” The buffer contained 150 mM KCl, 50 mM TEA (pH 7.8), 1 mM DTT, 100 μM UDP-glucose, various divalent cations (concentration in mM) or EDTA as indicated, and toxin A (100 μg/ml, black bars) or toxin B (88 μg/ml, stippled bars); reactions were incubated at 37 °C for 1 h. Each value represents the mean ± S.D. of at least three experiments.

ZnCl₂ supported some activity for both toxins, but had more effect on toxin A than toxin B. MnCl₂ not only supported toxin-mediated hydrolase activity, but was actually more efficient than MgCl₂ in this effect. Increasing the concentrations of either MgCl₂ or MnCl₂ to 10 mM did not enhance the hydrolase activities of toxin A or toxin B beyond the effects seen with 2 mM amounts of either cation.

Because Mn²⁺ markedly stimulated the hydrolase activity of the two toxins, we performed a series of experiments examining the effects of MnCl₂ on kinetic parameters of both toxins, similar to those performed for the KCl as outlined above. The time course of toxin B-mediated hydrolysis in the presence of Mn²⁺ is shown in Fig. 7. The time course for toxin A in the presence Mn²⁺ was comparable to that observed for toxin B, but with lower absolute percentages of substrate cleaved (data not shown). Consistent with the findings observed with the time-course experiments, substituting MnCl₂ for MgCl₂ in the assay buffer markedly increased the $V_{\text{max}}$ (mol of UDP-glucose hydrolyzed·mol of toxin⁻¹·h⁻¹) for toxin A from 17.3 (for MgCl₂) to 49.8 (for MnCl₂) (Fig. 8). For toxin B, a similar enhancement was observed; the $V_{\text{max}}$ increased from 89.7 (for MgCl₂) to 230.0 (for MnCl₂) (Fig. 9). Interestingly, the $K_m$ for UDP-glucose (expressed in μM) decreased for both toxins when Mn²⁺ was present in the buffer. For toxin A, the $K_m$ for UDP-glucose decreased from 142.3 μM (with MgCl₂) to 121.5 μM (with MnCl₂) and for toxin B, the $K_m$ for UDP-glucose decreased from 175.5 μM (for MgCl₂) to 89.4 μM (for MnCl₂).

**DISCUSSION**

*C. difficile* toxins A and B are UDP-glucose glucosyltransferases that monoglucosylate Rho small GTPases intracellularly (15). Toxin A had previously been shown to possess UDP-glucose hydrolase activity, but little was known about the kinetics, optimal reaction conditions, or potential mechanisms of activation of this toxin-mediated hydrolase activity (5). In this report, we demonstrated that toxin B also has significant UDP-glucose hydrolase activity, determined the kinetic parameters of this hydrolase reaction for both toxins, and characterized several factors required to permit and enhance toxin activity.
The hydrolase activity varied from 15.62 to 2000 nM, and the mediated UDP-glucose hydrolase activity.
The hydrolase assay was performed as described under “Experimental Procedures.” The buffer contained 150 mM KCl, 50 mM TEA (pH 7.8), 1 mM DTT, UDP-glucose (concentrations of 20, 100, and 500 μM, respectively), and toxin B (88 μg/ml); reactions were incubated at 37 °C for the times indicated in the graph. Each value represents the mean ± S.D. of at least two separate experiments. Results of experiments using toxin A were similar (data not shown).

Both toxins appear to follow Michaelis-Menten kinetics for the hydrolysis of UDP-glucose (Fig. 2). The Michaelis constants for UDP-glucose (K_m) were similar for each toxin. The K_m values for UDP-glucose are comparable to the intracellular concentration for UDP-glucose of ~100 μM and are similar to the K_m values reported for other bacterial UDP-glucose hydrolases (16). For example, the UDP-glucose hydrolase of Escherichia coli has a K_m for UDP-glucose of ~200 μM (17). Intriguingly, we found that the maximal velocity of the hydrolase reaction mediated by the two toxins was significantly different, with toxin B being about 5 times more active than toxin A. The toxins are known to differ in their abilities to cause cytotoxicity in vitro (toxin B is more active), but our present study demonstrates significant and quantitative differences in the enzymatic activities of the two toxins (3). The differences in cytotoxic potencies of toxins A and B in vitro appear to be cell-type specific and in some cases may reflect different rates of toxin-binding or cellular uptake (18–20). It is also possible that part of the different biological effects of the toxins could be a result of differences in their enzymatic activities (20, 21).

Toxins A and B exhibit similar overall structural homology. Toxins A and B are 45% identical and 63% conserved when amino acids sequences are compared (22, 23). Belonging to a family of large clostridial toxins, C. difficile toxins A and B have three similar structural domains: an NH₂-terminal region believed to contain the glucosyltransferase activity, a middle hydrophobic domain which may be involved in membrane translocation, and a COOH-terminal region of repeating sub-units involved in receptor-binding (24). The amino-terminal 546 amino acids of toxin B has recently been shown to contain catalytic regions for hydrolase and transferase activities (16). Interestingly, we found that the maximal velocity of the hydrolase reaction mediated by the two toxins was significantly different, with toxin B being about 5 times more active than toxin A. The toxins are known to differ in their abilities to cause cytotoxicity in vitro (toxin B is more active), but our present study demonstrates significant and quantitative differences in the enzymatic activities of the two toxins (3). The differences in cytotoxic potencies of toxins A and B in vitro appear to be cell-type specific and in some cases may reflect different rates of toxin-binding or cellular uptake (18–20). It is also possible that part of the different biological effects of the toxins could be a result of differences in their enzymatic activities (20, 21).

There are essential biochemical similarities between hydrolyase and glucosyltransferase reactions. We can speculate, therefore, that detailed kinetic analysis of the characteristics and mechanisms of activation of the toxin-mediated glucosyltransferase reactions will reveal similarities to those observed for the hydrolase reaction. Indeed, the possibility that the toxin-mediated hydrolase reaction itself may contribute to pathogenesis cannot currently be completely excluded nor can the hypothesis that hydrolase activity represents only uncoupled transferase activity. We are currently working to develop a transferase-deficient, but hydrolase-competent, toxin mutant would help address this issue.

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3 This program (version 9.2) is available via the World Wide Web from the ExPaSy server at the Fred Hutchinson Cancer Center (http://blocks.fhrc.org).
nizes a consensus pattern amino acid sequence present in the glycosylhydrolase family of enzymes (25). The toxin A sequence score for this block was in the 98th percentile with a p value of ~0.01. The homology between toxin A and a representative family member (endo-exoglucanase B of Caldocoecum saccharolyticum) is shown (Structure 1).

The shaded glutamate residue in Structure 1 is known to be the active site in this class of enzymes; it participates directly in glycosidic bond cleavage by acting as a nucleophile (25). The corresponding sequence in toxin B is only 33% identical to that found in toxin A, and the glutamate is replaced by a aspartate residue. Such differences in this putative active site could potentially account, at least in part, for differences seen in the hydrolyase activities of the toxins. We are currently examining potentially account, at least in part, for differences seen in the hydrolyase activities of the toxins. We are currently examining residues in this putative active site of toxins A and B. In preliminary experiments, we have found that a recombinant fusion protein containing the first 562 amino acid residues of toxin A displays both hydrolyase and transferase activities and that these activities appear to be equal to, or even greater than, those of holotoxin A. As mentioned, previous reports suggest that the transferase domain of toxin B is also contained within the amino-terminal one-third of the molecule.

Our experiments indicate that toxins A and toxin B require the presence of potassium as a cofactor for optimal UDP-glucose hydrolysis and that both exhibit a potassium-dependent enhancement of activity. With toxin B, for example, the hydrolyase activity increased ~50-fold as the potassium concentration was increased from 0 to 150 mM (Fig. 3). The normal intracellular level of KCl is ~150 mM; therefore, exposure to intracellular potassium may represent one form of toxin activation upon transfer from the extracellular to the intracellular environment. The mechanism whereby the potassium may augment the activity of the toxins remains unknown, but potassium-requiring enzymes have been described in both prokaryotic and eukaryotic organisms (26, 27). For mammalian pyruvate kinase, it is believed that potassium ions alter the overall active conformation of the enzyme into a more favorable state and may also directly participate directly in catalysis by coordinating the alignment of specific active groups at the catalytic site (28, 29). It is interesting to note both pyruvate kinase and the C. difficile toxins display similar responses to increased concentrations of potassium: increased $V_{max}$ with little change in the $K_m$ for the substrate (30).

In our assay, both toxins required certain divalent cations for activity and were inactive in the presence of EDTA. Most glycosyltransferases or glycohydrolases that use nucleotide hexoses require divalent cations for activity; the cation associates with the negatively charged phosphate groups to facilitate binding of the complex to the enzyme (17, 31, 32). The particular divalent cation that permits optimal activity depends on the specific enzyme system being studied. For toxin A and toxin B, manganese was the most potent at stimulating toxin activity and, interestingly, also decreased the Michaelis constant for the complex to the enzyme (17, 31, 32). The partic-

other nucleotide-binding proteins and enzymes also exhibit this in vitro preference for manganese over magnesium (33–35). In most instances, the in vivo significance of this result is difficult to ascertain. For example, the intracellular concentration of manganese is usually much less than that of magnesium; however, relative subcellular differences in the concentration of these cations may exist in cells, and, in these instances, regulation by manganese could be physiological relevant (33).

The detailed kinetic analyses of the UDP-glucose hydrolyase activities of C. difficile toxins A and B provide important new information that is valuable in understanding the characteristics and activation mechanisms of UDP-glucose hydrolases. In addition, these findings provide insight into potential regulatory mechanisms for the toxins and also are likely relevant to the glycosyltransferase reactions catalyzed by these toxins. Future work in this field will undoubtedly focus on identifying the specific active sites within each toxin and addressing the relevance of these enzymatic activities to the pathophysiolog

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