Calcium Binding by the N-Terminal Cellulose-Binding Domain from *Cellulomonas fimi* β-1,4-Glucanase CenC†

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**ABSTRACT:** The interaction of the N-terminal cellulose-binding domain, CBDN1, from *Cellulomonas fimi* β-1,4-glucanase CenC with calcium was investigated using NMR spectroscopy and calorimetry. CBDN1 binds a single calcium ion with an equilibrium association constant of approximately 10^4 M⁻¹ at 35 °C and pH 6.0. Binding is exothermic (−42 ± 2 kJ mol⁻¹) under these conditions and is accompanied by a small negative change in heat capacity (∆Cp = −0.41 ± 0.16 kJ mol⁻¹ K⁻¹). From an NMR line shape analysis, the rate constants for calcium association and dissociation were found to be (5 ± 2) × 10² s⁻¹ M⁻¹ and (4.5 ± 0.6) × 10² s⁻¹, respectively. The rapid association kinetics indicate that the calcium-binding site on CBDN1 is accessible and, to the first approximation, preformed. Based on patterns of chemical shift perturbations, and structural comparisons with the *Bacillus* sp. 1,3-1,4-β-glucanases, the backbone carbonyl oxygens of Thr8, Gly30, and Asp142 and a side chain carboxyl oxygen of Asp142 are postulated to form the calcium-binding site of CBDN1. Consistent with the calcium-independent affinity of CBDN1 for cellopentaose, this exposed site is located on the face of CBDN1 opposite to that forming the oligosaccharide-binding cleft. The midpoint denaturation temperature of CBDN1 is increased by approximately 8 °C at pH 6.0 in the presence of saturating amounts of calcium, confirming that metal ion binding is thermodynamically linked to native-state stability.

Enzymes that degrade cellulose often contain one or more domains that mediate binding to this complex substrate (1). Over 200 putative cellulose-binding domains (CBDs)¹ have been identified and classified into 13 families based on their sequence similarity (2). In particular, the N-terminal family IV CBD from *Cellulomonas fimi* β-1,4-glucanase CenC (CBDN1) has been extensively studied. CBDN1 has a unique specificity in that it binds to amorphous cellulose, soluble cellooligosaccharides, and a variety of soluble glucans, but not to crystalline cellulose (3–5). The structure of CBDN1 is comprised of two five-stranded β-sheets that fold into a jellyroll β-sandwich (6). The oligosaccharide-binding site is formed by a cleft that runs across one β-sheet face of the protein. The presence of this cleft, into which single stands of sugar can lie, explains the selectivity of CBDN1 for amorphous cellulose. In contrast, CBDs that interact with crystalline cellulose have a flat binding surface with three solvent-exposed aromatic side chains (7–9).

During a previous NMR study of oligosaccharide-free CBDN1, it was noted that resonances from 13 amides at its N-terminus and near its single disulfide bond (Cys33–Cys140) were not observed in a 1H–15N HSQC spectrum due to severe exchange broadening (4). Signals from these amides were detected only upon addition of excess cellotetraose, but not any other cellooligosaccharide. Subsequent investigations revealed that the cellotetraose was contaminated by a substance that bound to CBDN1 and caused the exchange-broadened amide resonances to appear. At the same time, we noted that CBDN1 shares a common fold with a number of other proteins including the 1,3-1,4-β-glucanases from *Bacillus* sp. (10–12), and the CBDs from *Clostridium thermocellum* scaffoldin subunit Cip A (CBDCip⁹) and *Thermomonospora fusca* endoxoglucanase E4 (CBDE4; 13). Each of these proteins was found to contain a bound calcium ion. We therefore suspected that CBDN1 also binds calcium

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¹ Abbreviations: CBDCip, cellulose-binding domain from *Clostridium thermocellum* scaffoldin subunit Cip B; CBDE4, cellulose-binding domain from *Thermomonospora fusca* endoxoglucanase E4; CBDN1, N-terminal cellulose-binding domain from *Cellulomonas fimi* β-1,4-glucanase CenC; CBDN1-Ca⁴⁺, 1:1 complex of CBDN1 and calcium; CBDN2, cellulose-binding domain from *Cellulomonas fimi* β-1,4-glucanase CenC following CBDN1 in sequence; CBDN2E2, tandem cellulose-binding domain from *Cellulomonas fimi* β-1,4-glucanase CenC; HSQC, heteronuclear single quantum correlation; pH₅, observed pH meter reading without correction for isotope effects; Tₘ, midpoint temperature of the unfolding transition.
Calcium Binding by CBD$_{N1}$

and that the sample of celloctetraose was contaminated with this metal ion.

Using NMR and calorimetry, we have confirmed that CBD$_{N1}$, both as an isolated domain and in tandem with the second CBD from CenC (CBD$_{N1N2}$), binds calcium and other divalent cations. The stability of CBD$_{N1}$ is increased in the presence of calcium, while its affinity for cellopentaose remains unaffected. This paper reports a detailed structural, thermodynamic, and kinetic analysis of the interactions of CBD$_{N1}$ and CBD$_{N1N2}$ with this metal ion.

MATERIALS AND METHODS

Sample Preparation. Samples of unlabeled and 99 % $^{15}$N-labeled CBD$_{N1}$ were prepared as described previously (4, 6). Samples of CBD$_{N2}$ and CBD$_{N1N2}$ were prepared in a similar manner as CBD$_{N1}$, and will be described in detail elsewhere. To remove metal ions, all buffers were passed over a Chelex-100 column, and all plasticware, glassware, and NMR tubes were soaked overnight in 4 M HCl and then thoroughly rinsed with deionized water. Metal ions were removed from the protein samples by the addition of excess EDTA, followed by extensive buffer exchange using a Microsep concentrator or by size-exclusion HPLC with a Waters Protein-Pak 125 column. Protein concentrations were determined using measured ε$_{280}$ values of 21 370 M$^{-1}$ cm$^{-1}$ (CBD$_{N1}$), 20 500 M$^{-1}$ cm$^{-1}$ (CBD$_{N2}$), and 40 740 M$^{-1}$ cm$^{-1}$ (CBD$_{N1N2}$).

NMR Spectroscopy. The buffer used for the NMR experiments presented in this study was 50 mM sodium chloride, 50 mM sodium $[^{1}H] $acetate, 0.02% sodium azide, 10% D$_2$O/90% H$_2$O at pH* 6.0. Acetate binds calcium weakly, with a $K_s$ of ca. 100 M$^{-1}$ (Brian Sykes, personal communication), and thus does not compete significantly with CBD$_{N1}$. Experiments were performed on a Varian Unity 500 MHz spectrometer equipped with a triple-resonance probe and pulsed field gradients. Enhanced-sensitivity pulsed field gradient $^{1}H$--$^{15}$N HSQC experiments with selective water flip-back pulses (15) were recorded with uniformly $^{15}$N-labeled proteins. All spectra were recorded at 35 °C and analyzed using a combination of NMRPipe, NMRDraw (14), and FELIX (Biosym Technologies).

Determination of Binding Constants by NMR Spectroscopy. The binding of calcium to CBD$_{N1}$ and CBD$_{N1N2}$ at 35 °C and pH* 6.0 was measured quantitatively using $^{1}H$--$^{15}$N NMR spectroscopy. A 0.5 M stock solution of CaCl$_2$ was prepared by weight from the anhydrous salt in the same buffer as used for the protein samples. The pH* was maintained at 6.0. Ten $^{1}H$--$^{15}$N HSQC spectra of CBD$_{N1}$ (initially 1.4 mM) with increasing concentrations of calcium (final concentration 7.1 mM) were acquired as 512 × 96 complex points in the $^{1}H$ and $^{15}$N dimensions with spectral widths of 7000 and 1650 Hz, respectively. Fourteen $^{1}H$--$^{15}$N HSQC spectra of CBD$_{N1N2}$ (initially 0.48 mM) with increasing concentrations of calcium (final concentration 1.5 mM) were acquired using 512 × 96 complex points in the $^{1}H$ and $^{15}$N dimensions with spectral widths of 6500 and 1450 Hz, respectively.

Equilibrium association constants were determined by nonlinear least-squares fitting of the chemical shift data versus the total added concentration of calcium or cellopentaose to the Langmuir isotherm describing the binding of one ligand molecule to a single protein site (4). The data were fit using the program PLOTDATA (TRIUMF, UBC, Vancouver).

Determination of Binding Constants by Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) was performed using a Microcal MCS ITC. All samples were pH 6.0 in 50 mM sodium acetate and 50 mM sodium chloride, except in one case where sodium was replaced by tetramethylammonium ion as the buffer cation. Titrations were performed by injecting 26 consecutive 10 µL aliquots of a stock CaCl$_2$ solution into the ITC cell (volume = 1.528 mL) containing calcium-free CBD$_{N1}$. Five independent titration experiments were performed at 35 °C to determine the binding constant for calcium by CBD$_{N1}$. Binding stoichiometry, enthalpy, and equilibrium association constants were determined by fitting the data, corrected for the heat of dilution of the titrant, to a 1:1 bimolecular interaction model. Three additional titrations were performed at 25, 30, and 40 °C to measure the heat capacity of binding.

Differential Scanning Calorimetry. Differential scanning calorimetry (DSC) was performed with a NanoDSC (Calorimetry Sciences Corp.) using a scanning rate of 1.0 K min$^{-1}$. Two CBD$_{N1}$ samples in 50 mM cacodylate buffer, pH 6.0, were analyzed, either in the presence of a 100-fold molar excess of EDTA (apo-CBD$_{N1}$) or in the presence of a 100-fold molar excess of CaCl$_2$ (CBD$_{N1}$•Ca$^{2+}$). DSC thermograms measurements and simulations were executed according to the method of Creagh et al. (16). To examine possible buffer effects, experiments were repeated with CBD$_{N1}$ in 50 mM sodium acetate and 50 mM sodium chloride at pH 6.0 using a Model 4215 MC-DSC (Calorimetry Sciences Corp.).

Analysis of Binding Kinetics. Calcium association/dissociation kinetics were determined by line shape analysis of the $^{1}H$ and/or $^{15}$N signals from amides showing intermediate two-site chemical exchange during the titration of CBD$_{N1}$ with this metal ion. This analysis was done manually by matching the experimental line shape and frequency of selected resonances with those from simulated spectra based on the formalism described by Sandström (17). The experimental 1D $^{1}H$ and 2D $^{1}H$--$^{15}$N HSQC spectra were processed using only a 2 Hz line broadening window function. Spectral simulations were obtained using values for the fraction of protein bound ($f_b$), the total change in chemical shift of a given nucleus between the bound and free forms ($\Delta \delta_{\text{bound}}$), the dissociation rate constant ($k_{\text{off}}$), and the transverse relaxation times for the $^{1}H$ or $^{15}$N nucleus in the free ($T_{2b}$) and bound ($T_{2b}$) state. Values of $f_b$ were calculated using the previously determined equilibrium binding constant ($K_e$) for CaCl$_2$ by CBD$_{N1}$. Values of $T_{2b}$ were estimated from the total line widths at half-height, and checked by comparison of the experimental and calculated line shapes for free (apo-CBD$_{N1}$) and fully calcium-saturated protein (CBD$_{N1}$•Ca$^{2+}$), respectively. Spectra were then simulated as a function of $k_{\text{off}}$ using a program written by Michael Strain (University of Oregon) that runs...
as a macro in FELIX v2.3. The association rate constant was then calculated from the relationship 

\[ k_{on} = k_{off}K_a \]

RESULTS

CBDN1 Binds Divalent Metal Ions. The binding of CBDN1 to a series of diamagnetic metal ions (Ca\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), and Mg\(^{2+}\)) was screened by one-dimensional NMR spectroscopy. Based upon the numerous perturbations of the spectrum of CBDN1 resulting from the addition of each ion, including changes in the chemical shifts and line widths of the downfield peaks from Gly7 H\(N\) and Trp16 H\(A\), we conclude that all four metals are bound by this protein (Figure 1A). Qualitatively, Ca\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), and Mg\(^{2+}\) produce similar changes in the spectrum of CBDN1, indicating that each binds at the same location in this CenC domain. However, the magnitudes of the spectral changes resulting from the addition of 10-fold molar excess of each metal to the same concentration of protein differ, and thus the apparent affinity of CBDN1 for these divalent cations decreases in the order 

\[ Ca^{2+} > Zn^{2+} > Cd^{2+} > Mg^{2+} \]

Comparable spectral perturbations were observed previously upon the addition of cellotetraose to CBDN1 (4), confirming that the original sample of sugar was indeed contaminated with metal ions.

Calcium Binding: Stoichiometry and Association Constant. After the initial screen for metal binding, the interaction of calcium with CBDN1 was studied in detail using one- and two-dimensional NMR spectroscopy (Figures 1B and 2). Assignments of the resonances from the amide \( ^1H \) and \( ^15N \) nuclei in apo-CBDN1 and CBDN1-Ca\(^{2+}\) were obtained using a suite of multidimensional heteronuclear correlation experiments, combined with monitoring the chemical shifts of these groups as a function of added calcium (4, and unpublished data). Based on the chemical shifts of peaks, such as those arising from Trp16 H\(A\) and Gly7 H\(N\), it is estimated that without steps taken to remove metal ions, freshly prepared samples of CBDN1 are approximately 20% calcium-loaded. The absence of resonances from 13 amides in the spectrum of untreated CBDN1 results from line broadening due to calcium binding on the time scale of intermediate chemical exchange. Addition of EDTA leads to the appearance of all but five of these peaks at chemical shifts corresponding to the metal-free state of the protein (Figures 1 and 2). Based on a detailed \( ^15N \) relaxation study of CBDN1 (in preparation), the resonances from the amides of Gly5, Thr8, Ala68, Val144, and Leu146 most likely remain unidentified in apo-CBDN1 due to conformational line broadening.

The changes in normalized \( ^1H \) and \( ^15N \) chemical shifts of 14 amides in CBDN1 versus added calcium are shown in Figure 3. These amides exhibit fast exchange on the NMR chemical shift time scale between the free and calcium-bound forms of this protein, and, as evident from their coincident titration curves, are all affected by the same binding event.
Based on the observed plateau in these curves at a metal-to-protein ratio of 1:1, we conclude that CBD N1 binds a single calcium ion. The equilibrium constant ($K_a$) for the association of calcium to this site was determined by a nonlinear least-squares fit of the $^1$H-$^{15}$N HSQC chemical shift data to a simple Langmuir isotherm. For each amide, two $K_a$ values were obtained, one using the data for the $^1$H N and the other for the $^{15}$N nucleus. In total, 45 fits for 28 different amides (Asp10, Gly12, Gly15, Trp16, Thr29, Gly30, Ala31, Ala53, Arg63, Thr65, Ala66, Ser69, Asp71, Arg100, Leu139, and Leu141) versus the total concentration of added CaCl$_2$. The coincident titration curves indicate that the amides all monitor the same binding event. The arrow marks the plateau where the total calcium concentration equals the total protein concentration ($\sim$1.4 mM), demonstrating a stoichiometry of 1:1. The solid lines are the best fits of the data to the Langmuir isotherm describing the binding of a single calcium ion to CBD$_{N1}$.

**FIGURE 2**: Titration of CBD$_{N1}$, CBD$_{N2}$, and CBD$_{N1N2}$ with CaCl$_2$ monitored by $^1$H-$^{15}$N HSQC spectroscopy. This figure illustrates portions of the overlaid spectra of these three proteins in the presence of increasing amounts of calcium up to a 3–5-fold molar excess. Arrows indicate the directions in which the amide $^1$H-$^{15}$N peaks shift upon addition of the metal ion. For the sake of clarity, only selected peaks are assigned. Peaks from the amides of the isolated CBD$_{N2}$ and from the CBD$_{N2}$ portion of the tandem CBD$_{N1N2}$ are identified by shading. Based on the observed chemical shift perturbations, it is evident that CBD$_{N1}$, both in isolation or as part of the tandem CBD$_{N1N2}$, binds calcium, whereas CBD$_{N2}$ does not.

Based on the observed plateau in these curves at a metal-to-protein ratio of 1:1, we conclude that CBD$_{N1}$ binds a single calcium ion. The equilibrium constant ($K_a$) for the association of calcium to this site was determined by a nonlinear least-squares fit of the $^1$H-$^{15}$N HSQC chemical shift data to a simple Langmuir isotherm. For each amide, two $K_a$ values were obtained, one using the data for the $^1$H N and the other for the $^{15}$N nucleus. In total, 45 fits for 28 different amides (Asp10, Gly12, Gly15, Trp16, Thr29, Gly30, Ala31, Gly39, Val48, Leu49, Gly51, Val52, Ala53, Arg63, Thr65, Ala66, Ser69, Asp71, Thr73, Leu95, Ser97, Arg100, Val102, Thr105, Leu139, Leu141, and Ala145) were determined, yielding an average $K_a$ of 1.1 × 10$^5$ M$^{-1}$ with a standard deviation of 0.5 × 10$^5$ M$^{-1}$ at 35 °C and pH 6.0.

The association of calcium with CBD$_{N1}$ was also investigated using isothermal titration calorimetry (Figure 4). Analysis of calcium binding by CBD$_{N1}$ using isothermal titration calorimetry. (A) Raw titration data showing the heat response resulting from each 10 µL injection of 11 mM CaCl$_2$ into the ITC cell containing 0.8 mM CBD$_{N1}$ in 50 mM sodium acetate and 50 mM sodium chloride at pH 6.0 and 35 °C. (B) Peak area normalized to the moles of CaCl$_2$ added and corrected for the heat of dilution (squares), and the nonlinear least-squares fit (line) to a Langmuir isotherm. The average of the best fits for 5 titrations using different titrant and titrand concentrations gives $n = 1.05 \pm 0.04$ calcium ions per CBD$_{N1}$, $K_a = (1.0 \pm 0.2) \times 10^5$ M$^{-1}$, and $\Delta H_a = -42 \pm 2$ kJ mol$^{-1}$. Analysis of the ITC data demonstrates that the stoichiometry of binding is 1:1 ($n = 1.05 \pm 0.04$) and that the association constant $K_a$ is (1.0 ± 0.2) × 10$^5$ M$^{-1}$ at pH 6.0 and 35 °C in 50 mM sodium acetate and 50 mM sodium chloride. Therefore, the results obtained by spectroscopic and calorimetric methods are in excellent agreement. The enthalpy of association (binding) $\Delta H_a$ is $-42 \pm 2$ kJ mol$^{-1}$ under these conditions. The binding enthalpy was also measured as a function of temperature from 25 to 40 °C (data not shown). Between 25 and 35 °C, a linear relationship exists, the slope of which gives a heat capacity change upon calcium binding, $\Delta C_p$, of $-0.41 \pm 0.16$ kJ mol$^{-1}$ K$^{-1}$. As shown...
in the DSC thermogram for apo-CBD$_{N1}$ (Figure 6), the protein is completely folded at temperatures below $\sim 33^\circ$C. However, at 40 $^\circ$C, a significant fraction of the protein is denatured, and thus the ITC data measured at this temperature are associated with both the refolding of CBD$_{N1}$ and the binding of calcium. As a result, the measured enthalpy of association at 40 $^\circ$C deviates from the linear temperature dependence defining $\Delta C_{p,a}$.

To determine if displacement of sodium by calcium at the binding site of CBD$_{N1}$ contributes to the binding thermodynamics reported here, the ITC titration was repeated in tetramethylammonium chloride buffer at pH 6.0 and 35 $^\circ$C. The bulky cation of this salt should not associate to the metal-binding site in CBD$_{N1}$. Under these conditions, the $K_a$ increases by a factor of only 2. This change lies on the border of statistical significance in an ITC experiment where $K_a$ is on the order of $10^8$ M$^{-1}$. The binding enthalpy $\Delta H_a$ also remains unchanged at $-42 \pm 3$ kJ mol$^{-1}$. Together this indicates that any competing effects of the sodium ion are very small, and implies that the binding site is specific for divalent cations.

**Kinetics of Calcium Binding.** The kinetics of the association and dissociation of calcium and CBD$_{N1}$ were determined from a line shape analysis of nuclei, such as Trp16H$_{1}$ and Gly7 H$_{N}$, that exhibit intermediate exchange on the NMR time scale. As expected from the classical model of two-site chemical exchange, the signals from these nuclei appear as sharp peaks in apo-CBD$_{N1}$ and CBD$_{N1}$:Ca$^{2+}$, yet are severely broadened under conditions of partial saturation. Seven different line shapes from six residues in CBD$_{N1}$ were analyzed according to the formalism of Sandström (17). These were the $^1$H line shape of Trp16 H$_{1}$ obtained from one-dimensional spectra, and the $^1$H line shapes of Gly30, Ala31, Leu139, Leu141, and Ala145 and the $^{15}$N line shape of Gly30 obtained from $^1$H--$^{15}$N HSQC spectra collected during the titration of this protein with CaCl$_2$. Values for $k_{on}$ were obtained by simulation of the spectrum of each nucleus at as many of the titration points as possible to yield an average $k_{on}$ for each peak (Figure 5). From these individual points, a global average of $4.5 \times 10^2$ s$^{-1}$ with a standard deviation of $0.6 \times 10^2$ s$^{-1}$ was established for the dissociation rate constant of the CBD$_{N1}$:Ca$^{2+}$ complex. A value for $k_{off}$ of $(5 \pm 2) \times 10^7$ M$^{-1}$ s$^{-1}$ was then calculated from the experimentally determined $K_a$ and $k_{off}$ values.

**Effect of Calcium on Cellopentaose Binding by CBD$_{N1}$.** Calcium-saturated CBD$_{N1}$ was titrated with cellopentaose to determine if the presence of the metal ion alters the affinity of the protein for cellooligosaccharides. As was found with a sample of $\sim 20\%$ calcium-loaded CBD$_{N1}$ (4, 5), the stoichiometry of the CBD$_{N1}$:Ca$^{2+}$ complex with cellopentaose is 1:1. This is revealed by the coincident titration curves, with plateaus at a total cellopentaose concentration equal to $3.1\times 10^{-4}$ M.

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A comparison of the (A) experimental and (B) simulated line shapes of Trp16 H$_{1}$ is shown. The experimental data are an expanded region of the one-dimensional $^1$H NMR spectra presented in Figure 1B, with the fraction of total protein in the calcium-bound form indicated for each point in the titration. The simulated line shapes were produced using a $k_{on}$ value of $500$ s$^{-1}$, $T_2$ values of 0.02 s (free) and 0.015 s (bound), and a difference in frequency of 283 Hz between the free and calcium-bound states of CBD$_{N1}$.

**Effect of Calcium on the Thermodynamic Stability of CBD$_{N1}$.** Thermal denaturation of CBD$_{N1}$ follows a fully reversible two-state transition at all solution conditions examined in this study (16). Experimental thermograms are therefore well represented by the van’t Hoff equation. Figure 6 shows DSC thermograms for CBD$_{N1}$ at two extreme conditions of calcium loading. In the absence of calcium, reversible unfolding of apo-CBD$_{N1}$ occurs at a midpoint temperature of 46.7 $^\circ$C with a van’t Hoff unfolding enthalpy $\Delta H_u$ of 392.0 kJ mol$^{-1}$. With calcium ion bound at stoichiometric levels, the midpoint unfolding temperature of CBD$_{N1}$:Ca$^{2+}$ rises by approximately 8 $^\circ$C to 55.2 $^\circ$C. This represents a significant increase in protein thermal stability, such that at 35 $^\circ$C, the native state of CBD$_{N1}$ is stabilized by an additional $-14$ kJ mol$^{-1}$ of Gibbs energy. Consistent with the stabilizing effect of calcium, a midpoint unfolding temperature of 51.2 $^\circ$C at pH 6.1 was reported previously for CBD$_{N1}$ that, unknown at the time, was partially saturated.
Calcium Binding by CBDN1

Identification of the Calcium-Binding Site in CBDN1. As shown in Figure 2 and summarized in Figure 7, many amides in CBDN1 experience a change in their $^1$H$^N$ and/or $^{15}$N chemical shift upon calcium binding. When discussing these changes, it is important to note that chemical shift perturbations can arise either directly from the interaction of the nucleus with the ligand or indirectly from conformational changes resulting from the formation of a protein–ligand complex. Chemical shifts are also a very sensitive indicator of structure, with subtle conformational changes often leading to relatively large changes in shift. Keeping this in mind, it is possible to gain insight into the location of the metal-

with calcium (16). Note that although calcium has a pronounced effect on the thermal stability of CBDN1, its presence does not significantly change the thermodynamics of sugar binding reported in either Tomme et al. (5) or Creagh et al. (16). This is because the affinity of CBDN1 for cellulosiogosaccharides, such as cellopentaose, is independent of calcium and is not strongly temperature dependent ($\Delta C_p = -0.21 \text{ kJ mol}^{-1} \text{ K}^{-1}$, 5).

Calcium-loaded CBDN1-Ca$^{2+}$ is characterized by a total unfolding enthalpy $\Delta H_{\text{total}}$ of 518.2 $\text{kJ mol}^{-1}$ at 55.2 °C and pH 6.0. Identical energetics were measured with both 50 mM cacodylate and 50 mM acetate/50 mM sodium chloride buffers (i.e., those used in the DSC and ITC/NMR experiments, respectively; data not shown). This total unfolding enthalpy is the sum of the heats due to calcium dissociation and CBDN1 unfolding, $\Delta H_{\text{total}} = -\Delta H_a + \Delta H_u$. Using a measured $\Delta C_p,a$ of 7.5 $\text{kJ mol}^{-1} \text{ K}^{-1}$ for the unfolding of CBDN1 (16), $\Delta H_u$ at 55.2 °C is calculated to be 455.8 $\text{kJ mol}^{-1}$. Therefore, calcium dissociation contributes 62.4 $\text{kJ mol}^{-1}$ to the $\Delta H_{\text{total}}$ measured at 55.2 °C for CBDN1-Ca$^{2+}$.

This in turn corresponds to a calculated calcium-binding enthalpy $\Delta H_a$ of $-58.9 \text{ kJ mol}^{-1}$ at 46.7 °C and $-54.1 \text{ kJ mol}^{-1}$ at 35.0 °C, determined using the observed $\Delta C_p,a$ of $-0.41 \text{ kJ mol}^{-1} \text{ K}^{-1}$. This is in reasonable agreement with the exothermic binding enthalpy $\Delta H_a$ of $-42 \pm 2 \text{ kJ mol}^{-1}$ measured at 35 °C by ITC. Figure 6 also shows a predicted unfolding thermogram for CBDN1-Ca$^{2+}$ based on the thermodynamics of calcium binding measured by ITC, the DSC thermogram for apo-CBDN1, and the two-dimensional global minimization model of Creagh et al. (16). The predicted thermogram and van’t Hoff enthalpy are in close agreement with the DSC results measured for CBDN1-Ca$^{2+}$, indicating a consistent quantitative linkage between calcium binding and protein stability.

Identification of the Calcium-Binding Site in CBDN1. As shown in Figure 2 and summarized in Figure 7, many amides in CBDN1 experience a change in their $^1$H$^N$ and/or $^{15}$N chemical shift upon calcium binding. When discussing these changes, it is important to note that chemical shift perturbations can arise either directly from the interaction of the nucleus with the ligand or indirectly from conformational changes resulting from the formation of a protein–ligand complex. Chemical shifts are also a very sensitive indicator of structure, with subtle conformational changes often leading to relatively large changes in shift. Keeping this in mind, it is possible to gain insight into the location of the metal-

Figure 6: DSC thermograms for the unfolding of apo-CBDN1 (100-fold molar excess EDTA; $T_m = 46.7$ °C and $\Delta H_u = 392.0 \text{ kJ mol}^{-1}$) and calcium-saturated CBDN1-Ca$^{2+}$ (100-fold molar excess CaCl$_2$; $T_m = 55.2$ °C and $\Delta H_{\text{total}} = 518.2 \text{ kJ mol}^{-1}$) in 50 mM cacodylate buffer at pH 6.0. The circles are the experimental data, while the solid lines represent the nonlinear least-squares fit to a two-state model of unfolding. The dashed line is the curve predicted for CBDN1-Ca$^{2+}$ using the enthalpy of unfolding measured for calcium-free CBDN1, along with the calcium-binding enthalpy $\Delta H_a (-42 \text{ kJ mol}^{-1})$ and heat capacity change $\Delta C_p,a (-0.41 \text{ kJ mol}^{-1} \text{ K}^{-1})$ obtained from ITC studies.

Figure 7: (A) Identification of the metal-binding site in CBDN1 based on the observed patterns of chemical shift perturbation resulting from calcium binding. The absolute values of the differences between the $^{15}$N and $^1$H$^N$ chemical shifts (ppm) of the backbone amides in the free and calcium-bound forms of the protein are plotted as positive and negative numbers, respectively. The regions showing the greatest change in chemical shift involve the backbone amides in the free and calcium-bound forms of the protein are plotted as positive and negative numbers, respectively. The regions showing the greatest change in chemical shift involve the absence of added calcium (Gly5, Thr8, Ala68, V144, Leu146), identified in black. The top panel shows $\beta$-sheet B, which lies opposite to the oligosaccharide-binding face that is formed by $\beta$-sheet A (6). The molecule is rotated by 90° in the lower panel to provide a view through the binding cleft. Selected residues are labeled, and the amino and carboxyl termini are denoted by N and C, respectively. This figure was created using the program GRASP (33).
binding site in CBDN₁ from the patterns of amide chemical shift perturbations accompanying the addition of calcium to the protein (Figure 7A). To this end, the residues of CBDN₁ that experience the largest change in either \(^{13}N\) or \(^{1}H\) chemical shift, as well as those not detected in the absence of added metal (Gly5, Thr8, Ala68, Val144, Leu146), are identified on a worm diagram of CBDN₁ (Figure 7B).

Based on the data presented in this figure, it is clear that the chemical shifts of amides from five regions of CBDN₁ are strongly influenced by calcium binding. These include the N-terminus up to residue Val17, Gly30-Cys33 (β-strand B2), Thr65-Ala68 (β-strand B3), Leu139-Leu146 (β-strand B5), and Arg100 (β-strand B4). When mapped onto the tertiary structure of CBDN₁, these residues all lie on the face of the protein formed by the N-terminal amino acids and β-sheet B (6). In contrast, with the exception of Trp16 and Val17, no amides on the oligosaccharide-binding face (β-sheet A) of the protein show a significant change in chemical shift upon formation of the CBDN₁-Ca\(^{2+}\) complex.

The titration of calcium into CBDN₁ was also monitored by following chemical shifts of the methyl groups in a sample of 10% \(^{13}C\)-enriched protein (data not shown). The methyl peaks that are affected by calcium binding are those from Thr8\(^2\), Ile4\(^2\), Leu32\(^1\), Leu49\(^1\), Leu49\(^2\), and Ala68\(^2\), in order of decreasing chemical shift change. With the exception of Leu49, each of these residues is located in the N-terminal region of CBDN₁ or in β-sheet B.

Distilling the patterns of the calcium-dependent amide and methyl chemical shift perturbations shown in Figure 7, we conclude that the metal-binding site of CBDN₁ lies on the face of the protein formed by its N-terminus and β-sheet B. The changes in the resonances of the amides of Trp16 and Val17, located within the oligosaccharide-binding cleft of β-sheet A, are attributed to their proximity to the N-terminus of CBDN₁. The changes in the methyl resonances of Leu49 are explained as an indirect conformational effect of calcium binding due to its packing against the aromatic ring of Trp16.

**Calcium Binding by CBDN₂**

In the native enzyme CenC, CBDN₁ exists in tandem with a second family IV CBD (CBDN₂). The titrations of the isolated CBDN₂ and the combined CBDN₁N₂ with CaCl₂ were also studied using \(^{1}H\) and \(^{15}N\) HSQC spectroscopy (Figure 2). These data clearly reveal that CBDN₂ does not bind calcium appreciably as its amide \(^{1}H\) and \(^{15}N\) resonances remain completely unperturbed upon addition of a 3-fold molar excess of this metal ion. The spectrum of CBDN₂ is also unchanged after treatment with EDTA, eliminating the possibility of a tightly bound calcium ion that copurifies with the protein (data not shown). In contrast, the \(^{1}H\) and \(^{15}N\) resonances from numerous amides in CBDN₁N₂ shift upon the addition of CaCl₂. Therefore, the tandem CBD does bind this metal ion.

The \(^{1}H\) and \(^{15}N\) HSQC spectrum of the 296 residue CBDN₁N₂ is almost identical to that obtained simply by superimposing the individual spectra of CBDN₁ and CBDN₂, indicating that the two domains are, to the first approximation, structurally independent. This allows for the nearly complete assignment of amide resonances from CBDN₁N₂ using results obtained for the two isolated binding domains. Based on these assignments, we conclude that the amides in CBDN₁N₂ whose chemical shifts are perturbed upon the binding of calcium are located within the N-terminal CBDN₁ portion of this molecule. Small differences in the magnitudes of these chemical shift perturbations for corresponding residues in the two proteins may reflect slight differences in experimental conditions or subtle effects of CBDN₂ on the structure of CBDN₁. Furthermore, a stoichiometry of 1:1 and a calcium association constant \(K_c\) of \((1.2 \pm 0.4) \times 10^2 \text{ M}^{-1}\) were determined for CBDN₁N₂ based on fitting the observed changes in the \(^{13}N\) and \(^{1}H\) chemical shifts of 14 amides to a Langmuir isotherm (data not shown). Within experimental error, this is equal to the \(K_c\) determined for CBDN₁ by both NMR and ITC methods. Therefore, whether in isolation or in context of CBDN₁N₂, the N-terminal CBDN₁ binds a single calcium ion with an affinity that is not affected by the presence of its partner CBDN₂.

**DISCUSSION**

**Location of the Metal-Binding Site and the Structure of CBDN₁N₂-Ca\(^{2+}\)**.

Chemical shift perturbations provide a qualitative indicator of the location of the calcium-binding site on the face of CBDN₁ formed by its N-terminus and β-sheet B, but do not unambiguously identify the coordinating atoms. Therefore, the regions of CBDN₁ showing the greatest spectral change upon addition of calcium (Figure 7) were examined structurally for the presence of possible atoms that commonly interact with metal ions. Following this approach, two potential binding sites were identified. The first is the loop region, formed by residues 6–11, that contains Glu6, Asp10, and Asp11. In the minimized average structure of CBDN₁, the side chain of Glu6 points away from Asp10 and Asp11 (6). However, this region has the highest rms deviations in the structural ensemble, and thus the exact location, or locations, of these side chains may not be accurately defined. The second potential calcium-binding site includes Asp142 and Asp143 in β-strand B5.

In contrast to CBDN₁, CBDN₂ does not bind calcium. Thus, it is expected that the metal-ligating residues are not conserved between these two proteins. If the amino acid side chains comprising only one of two potential binding sites in CBDN₁ were conserved, this would provide compelling evidence for the identification of the calcium ligands. However, residues in both possible potential sites exhibit low sequence similarities. Specifically, Glu6, Asp10, and Asp11 in CBDN₁ correspond to a histidine, a serine, and a glutamate in CBDN₂, while Asp142 and Asp143 in CBDN₁ align with a serine and a glutamine in CBDN₂. This does not differentiate the two sites, but it is consistent with the lack of calcium binding by CBDN₂.

Strong evidence for the location of the calcium-binding site comes from a comparison of the structure of CBDN₁ with those of the Bacillus sp. 1,3-1,4-β-glucanases. As discussed previously (6), these proteins share a common jellyroll β-sandwich fold. In the hybrid Bacillus 1,3-1,4-β-glucanase H(A16-M), calcium is bound octahedrally, using Pro9 O, Gly45 O, Asp207 O, Asp207 O', and two water molecules as the coordinating ligands (10, 18, 19). The Bacillus licheniformis and Bacillus macerans 1,3-1,4-β-glucanase structures use the same ligands to coordinate calcium, but bind with pentagonal-bipyramidal geometry due to the involvement of three water molecules (11, 12). The calcium-binding sites of the 1,3-1,4-β-glucanases are remarkably similar in sequence and structure to the corresponding regions of CBDN₁. For example, all have a bulge in the β-sheet.
the number of water molecules bound to the metal ion in the CBD\textsubscript{N1}-Ca\textsuperscript{2+} complex, and thus the exact geometry of the coordinating ligands shown in Figure 8 could not be determined.

**Thermodynamics of Calcium Binding to CBD\textsubscript{N1}**. CBD\textsubscript{N1} binds calcium with a $K_a$ of ca. 10$^4$ M$^{-1}$ at pH 6.0 and 35 °C, as determined by both NMR and ITC methods. Similar thermodynamic values ($K_a$, $\Delta H$) were measured in the presence of sodium or tetramethylammonium buffers, indicating that these monovalent cations do not significantly compete with calcium for CBD\textsubscript{N1}. The same association constant was also measured for CBD\textsubscript{N2} in the context of the tandem CBD\textsubscript{N1N2}, demonstrating that its affinity for calcium is not influenced appreciably by the presence of CBD\textsubscript{N2}. This is consistent with the apparent structural independence of the two neighboring CenC cellulose-binding domains. Finally, the calcium association constant of CBD\textsubscript{N1} is comparable to that determined for the hybrid *Bacillus* 1,3-1,4-\textbeta-glucanase [(1.0 ± 0.4) × 10$^5$ M$^{-1}$ at pH 6.0; 18]. This supports the proposal that the calcium-binding sites of CBD\textsubscript{N1} and the β-1,3-1,4-glucanase are structurally similar. By way of comparison, $K_a$ values reported for calcium-binding proteins range from >10$^{10}$ M$^{-1}$ for thermitase to ~10$^7$ M$^{-1}$ for concanavalin A (24), indicating that CBD\textsubscript{N1} has moderate affinity for this divalent metal ion.

Calcium binding by CBD\textsubscript{N1} is moderately exothermic with a $\Delta$H of −42 kJ mol$^{-1}$ and exhibits enthalpy−entropy compensation ($\Delta S = −40.6$ kJ mol$^{-1}$ K$^{-1}$ at 35 °C). Exothermic behavior is often observed in small-ion tight-binding events where coordination between the ion and the ligands is highly favorable. For example, the enthalpy of binding of calcium to EDTA is −29 kJ mol$^{-1}$ at 20 °C and a solution ionic strength of 0.1 M (25). It is tempting to argue that the formation of the CBD\textsubscript{N1}-Ca\textsuperscript{2+} complex is driven by enthalpically favorable interatomic interactions which are compensated for entropically by a reduction in the translational and rotational degrees of freedom of the ion and associated protein backbone and side-chain atoms relative to the fully solvated state. However, such an argument is complicated by the role of water. The partial molal desolvation entropy for the calcium ion in water at ambient temperature is 53 J mol$^{-1}$ K$^{-1}$ (26), indicating that it is a structure-making solute. Based on the group contribution method of Makhatadze and Privalov (27), the dehydration entropies at 25 °C estimated for the postulated calcium ligands in CBD\textsubscript{N1} are 35 J mol$^{-1}$ K$^{-1}$ (Thr8 O, Gly30 O, and Asp142 O) and 27 J mol$^{-1}$ K$^{-1}$ (Asp142 O$\delta$). Thus, although this analysis is crude, dehydration effects associated with calcium binding appear to be entropically favorable for both the metal ion and the protein ligands. The fact that overall the affinity of calcium for CBD\textsubscript{N1} is reduced by a net negative entropy change suggests the losses in calcium and protein degrees of freedom dominate desolvation effects. This in turn indicates that the protein adopts a more rigid structure upon binding calcium. Using $^{15}$N relaxation measurements, we have observed that residues at the N-terminus of apo-CBD\textsubscript{N1} and, to a lesser extent, near position 142 exhibit conformational mobility on a micro- to millisecond time scale that is eliminated in the presence of added calcium ions (in preparation).

It is noteworthy that calcium binding to proteins is not often associated with a net loss of entropy (24, 28, 29). A
striking example is seen with equine lysozyme, where binding is entropically driven and compensated by an unfavorable positive binding enthalpy (30). Thus, the relative importance of factors, such as the organization of the solvation layer and the role of conformational dynamics, in establishing the overall affinity for calcium can differ significantly between proteins.

**Kinetics of Calcium Binding to CBDN1.** The association rate constant for calcium binding of $(5 \pm 2) \times 10^7$ M$^{-1}$ s$^{-1}$ is within 2 orders of magnitude of the diffusion-controlled limit of $\sim 10^9$ M$^{-1}$ s$^{-1}$. Similar $k_a$ values have been reported for several regulatory EF-hand proteins (24). This implies that the calcium-binding site of CBDN1 is accessible and, to the first approximation, preformed. That is, no major structural rearrangements or conformational transitions are necessary in the native protein to properly orient the atoms that ligate calcium. This conclusion is consistent with three additional lines of evidence. First, in the structural model of CBDN1*Ca$^{2+}$ presented in Figure 8, the ligating atoms are exposed on the surface of CBDN1. Also, note that Gly30 is located in the loop between $\beta$-strands B1 and B2, and Asp142 is within $\beta$-strand B5. Thus, two of the three postulated calcium ligands contribute to the jellyroll $\beta$-sandwich fold of CBDN1. Second, the residues forming the nascent calcium binding site in apo-CBDN1 have $^1$H and $^15$N chemical shifts indicative of a structured conformation and not a random coil or disordered state (Figure 2; 31). Furthermore, chemical shift perturbations accompanying the formation of the CBDN1*Ca$^{2+}$ complex are localized to this binding site, rather than being global to the entire protein (Figure 7). Third, we have studied the effects of calcium and oligosaccharide binding on the dynamic properties of CBDN1 using $^3$H and $^15$N relaxation methods (in preparation). In summary, we have observed that amides contributing to the metal-binding site are generally well-ordered on the picosecond–nanosecond time scale, in both the free- and calcium-loaded forms of CBDN1. However, this region of apo-CBDN1 exhibits conformational exchange between most probably nativelylike structures on the micro- to millisecond time scale that become restricted upon calcium binding. This mobility leads to the line broadening seen for Gly7 $^1$H in Figure 1, and likely precludes the detection of resonances from the amides of Gly5, Thr8, Ala68, Val144, and Leu146 in the metal-free protein. We postulate that conformational plasticity of an approximately preformed binding site allows for the facile association of calcium with CBDN1.

A Structural Role for Calcium Binding by CBDN1. The apparent biological role of calcium binding by CBDN1 is to stabilize the folded structure of this protein domain. As determined by DSC, the midpoint denaturation temperature of CBDN1 increases by 8 °C in the presence of CaCl$_2$ at pH 6.0. It is tempting to ask whether calcium stabilizes the native structure of CBDN1 by binding to three amino acids (Thr8, Gly30, and Asp142) that are distant along the length of its polypeptide chain. However, there does not appear to be a general correlation between the affinity of a protein for calcium, which is thermodynamically linked to its stability, and the number of residues between the chelating atoms (23, 24). This is expected if the metal ion binds only to the folded state of the protein and does not induce a disorder–order transition. Nevertheless, it is intriguing to note that the postulate calcium-binding site in CBDN1 is adjacent to the disulfide bond between Cys33 and Cys140. This single disulfide is critical for the integrity of the native CBDN1, as its reduction leads to the complete unfolding of the protein (16). Studies of the combined effects of metal binding and cysteine oxidation on the stability of CBDN1 may prove worthwhile to define the possible linkage between these two factors. It is also of note that CBDN3, which does not bind calcium, has a higher midpoint denaturation temperature than either apo-CBDN1 or CBDN1*Ca$^{2+}$ (unpublished observation).

The role of calcium in stabilizing the structure of CBDN1 is consistent with the finding that the presence of this cation does not perturb significantly the $K_a$ of the protein for cellobiose. This is not surprising in light of the fact that the calcium-binding site is on the opposite side of the protein ($\beta$-strand B) as is the sugar-binding cleft ($\beta$-strand A; Figures 7 and 8). Any structural changes that result upon metal complexation are unlikely to affect residues within the binding cleft. A similar biological role for calcium binding is exhibited by the Bacillus 1,3-1,4-$\beta$-glucanases. This ion was found to increase the stability of seven native and hybrid 1,3-1,4-$\beta$-glucanases against thermal or chemical denaturation (18, 21–23). X-ray crystallographic studies reveal that calcium binds at a position distant from the active site cleft of each glucanase, and thus is unlikely to influence their catalytic activities. In contrast, proteins, such as lectins, that exhibit calcium-dependent affinity for sugar often utilize the metal ion directly to aid in carbohydrate binding (32).

In general, little is known about the role of metal ion binding by CBDs. A calcium ion has been identified in the crystal structure of CBD$_{CIP}$ (9), as well as that of CBD$_{E4}$ (13). As in the case of CBDN1, the metal ions are bound at sites distant from the putative cellulose-binding faces of each of these proteins. It is likely that calcium also serves to stabilize the structures of CBD$_{CIP}$ and CBD$_{E4}$ without directly influencing their affinity for cellulose. Based on these three examples, it is reasonable to suggest that metal binding is one possible mechanism to enhance the stability of cellulases in order to ensure that they remain folded when secreted into potentially harsh natural environments by cellulytic microorganisms.

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Calcium Binding by CBD\textsubscript{N1}


