Interaction of Polysaccharides with the N-Terminal Cellulose-Binding Domain of *Cellulomonas fimi* CenC. 1. Binding Specificity and Calorimetric Analysis†

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ABSTRACT: The carbohydrate-binding specificity of the N-terminal cellulose-binding domain (CBD_{N1}) from *Cellulomonas fimi* β-1,4-glucanase C (CenC) was investigated using affinity electrophoresis, binding assays and microcalorimetry in parallel with NMR and difference ultraviolet absorbance spectroscopy [Johnson, P. E., Tomme, P., Joshi, M. D., & McIntosh, L. P. (1996) *Biochemistry 35,* 13895−13906]. Binding of CBD_{N1} on insoluble cellulose is distinctly different from other cellulose-binding domains. CBD_{N1} binds amorphous cellulose (phosphoric acid-swollen) with high affinity ($K_r = 5.1$ L g^{-1}), binds Avicel weakly and does not bind highly crystalline bacterial or tunicin cellulose. Moreover, CBD_{N1} binds soluble cellooligosaccharides and β-1,4-linked oligomers of glucose such as hydroxyethylcellulose, soluble β-1,3-1,4-glucans from barley and oat, but has no affinity for α-1,4-, β-1,3-, or β-1,6-polymers of glucose. This is the first report of a cellulose-binding domain with strong and specific affinity for soluble glycans. The thermodynamics for binding of CBD_{N1} to oligosaccharides, soluble glycans, and phosphoric acid-swollen cellulose were investigated by titration microcalorimetry. At least four β-1,4-linked glucopyranosides are required to detect binding. For larger glucans, with five or more glucopyranoside units, the binding constants and standard free energy changes are virtually independent of the glucan chain length, indicating that cellopentaose completely fills the binding site. Binding is moderately strong with binding constants ranging from 3 200 ± 500 M^{-1} for cellotetraose, to 25 000 ± 3 000 M^{-1} for the larger sugars. The reactions are controlled by favorable standard free enthalpy changes which are compensated in a linear fashion by a significant decrease in entropy. A predominance of polar interactions such as hydrogen bonding together with van der Waals interactions provide the major driving forces for the binding event.

Many enzymes involved in the degradation of biomass, such as cellulases and hemicellulases, need to associate tightly and specifically with their insoluble substrates to enhance the efficiency of degradation (Tomme et al., 1995a). Typically, these enzymes have acquired a carbohydrate- or cellulose-binding domain (CBD)[1] which facilitates this interaction (Béguin & Aubert, 1994; Gilkes et al., 1991; Tomme et al., 1995a) and, when removed, often reduces the catalytic activity on insoluble cellulose without affecting activity on soluble glycans (Gilkes et al., 1988; Tomme et al., 1988; Van Tilbeurgh et al., 1986).

To date, more than one-hundred-and-twenty putative cellulose-binding domains have been identified. They can be grouped in ten different families on the basis of their amino acid sequence (Tomme et al., 1995b). CBDs from different families are thought to have different properties, although CBDs belonging to the same family also may have subtle functional and structural differences (Din et al., 1994; Poole et al., 1993). The high diversity among CBDs therefore reflects the structural heterogeneity of the cellulose/hemicellulose matrix and is perhaps indicative of the specific requirements for hydrolysis of this structurally complex substrate.

It remains difficult to ascribe a precise role to CBDs due to the limited understanding of the mechanism of binding, conflicting reports on the properties of the CBDs, and incomplete characterization of the biological function. It is clear that CBDs can enhance the activity of polysaccharidases by increasing the local enzyme concentration on the substrate surface or by disrupting non-covalent interactions, thereby increasing substrate accessibility (Knowles et al., 1988; Din et al., 1991). Either mechanism could involve targeting of enzymes to distinct regions of the substrate by CBDs with different specificities.

Most CBDs bind both crystalline and amorphous cellulose, albeit with different affinities (Gilkes et al., 1992; Ong et al., 1993). Others, such as the CBD from the *C. fimi* xylanase D, are more specific and bind only crystalline cellulose (Millward-Sadler et al., 1994) or have affinities for insoluble xylan (Millward-Sadler et al., 1994) or chitin

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[1] Abbreviations: CBD, cellulose-binding domain; CBD_{N1}, N-terminal cellulose-binding domain from the *C. fimi* β-1,4-glucanase CenC; CBD_{N1N2}, tandem-repeated N-terminal cellulose-binding domains from the *C. fimi* β-1,4-glucanase CenC; CBD_{Cex}, C-terminal cellulose-binding domain from the *C. fimi* mixed exoglucanase/xylanase Cex; ITC, isothermal titration calorimetry; PASC, phosphoric acid-swollen cellulose; CMC, carboxymethylcellulose; HEC, hydroxyethylcellulose; DP, degree of polymerization; DS, degree of substitution; SDS, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; BSA, bovine serum albumin; AE, affinity electrophoresis.

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(Goldstein et al., 1993; Ong et al., 1993; Tomme et al., 1994).

Structural data for CBD_{Cex} from *C. fimi* (Xu et al., 1995) and CBD_{CBHI} from *Trichoderma reesei* (Kraulis et al., 1989) combined with mutagenesis studies (Din et al., 1994; Poole et al., 1993; Reinikainen et al., 1992) have shown that these particular CBDs interact with cellulose through various solvent-exposed, aromatic residues, aligned on one flat face of the protein surface. These CBDs lack a carbohydrate-binding groove or cleft. Despite the large body of structural and functional information available, only the mechanism of interaction of CBD_{Cex} with crystalline cellulose is reasonably well characterized. This is in large part due to microcalorimetric analysis which has demonstrated that binding is driven by a large entropic change accompanying dehydration of both the protein and sugar polymer surfaces (Creagh et al., 1996).

The two family IV binding domains, CBD_{N1} and CBD_{N2}, present in tandem at the N-terminus of the β-1,4-glucanase C (CenC) from *C. fimi*, are unusual in that they bind to amorphous but not crystalline cellulose (Coutinho et al., 1992). CenC has a number of structural and functional features that distinguish it from related glycosidases. Along with the CBD_{N1} and CBD_{N2} in tandem at the N-terminus, two additional tandem domains (C1 and C2), of unknown function, are located at the C-terminus (Coutinho et al., 1991). Compared to the other *C. fimi* cellulases, CenC has a high enzymatic activity on soluble glucans, *i.e.*, carboxymethylcellulose, β-glucans, and amorphous cellulose but relatively low activity on the more crystalline substrates (Tomme et al., 1996). This trend in the activities seems, at least in part, linked to the properties of the two CBDs (Coutinho et al., 1993). Moreover, CenC is a more processive enzyme than most endoglucanases reported to date. This processivity may be mediated or enhanced by the CBDs (Tomme et al., 1996).

The novel sugar-binding properties of CBD_{N1} have motivated a full functional and structural analysis of this cellulose-binding domain with the aim of determining the forces involved in the carbohydrate−protein interactions and the specificity of these interactions. An accurate understanding of the relationship between structure, specificity, and affinity requires a complementary set of detailed structural and thermodynamic analyses. Thermodynamic studies are particularly valuable in determining the driving forces for the binding process, including contributions of hydrogen bonding and van der Waals interactions. Indeed, microcalorimetry has become one of the most powerful tools to study carbohydrate−protein interactions (Bains et al., 1992).

Using isothermal titration microcalorimetry, we report the thermodynamics and stoichiometry of the binding of small, soluble cellooligosaccharides and larger, soluble glucans to CBD_{N1}. Although many calorimetric studies have been described for lectins (Bains et al., 1992; Chervenak & Toone, 1995), carbohydrate-binding antibodies (Brummel et al., 1993; Sigurskjold & Bundle, 1992) and starch-binding domains (Sigurskjold et al., 1994) with their soluble sugar ligands, this is the first report of the binding and thermodynamics of soluble glycans to a cellulose-binding domain. The energetics for binding of CBD_{N1} to insoluble amorphous cellulose were also investigated. Combined, the results are used to determine the carbohydrate-binding specificities of CBD_{N1} for both soluble and insoluble polysaccharides. Finally, the functional properties of CBD_{N1} are interpreted within the context of cellulose degradation.

MATERIALS AND METHODS

Carbohydrates and Polysaccharides. Microcrystalline cellulose (Avicel PH101) was obtained from FMC International (Little Island, County Cork, Ireland). Bacterial microcrystalline cellulose (BMCC) was prepared from cultures of *Acetobacter xylinum* (ATCC 23769) as described previously (Gilkes et al., 1992). Regenerated cellulose (PASC) was obtained by phosphoric-acid treatment of Avicel PH101 as reported previously (Coutinho et al., 1992). Tunicin cellulose (Ceolus Cream FP-03) was obtained from Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). Carboxymethylcellulose (CMC, sodium salt, low-viscosity grade, nominal DP 400, nominal DS 0.7), hydroxypropylmethylcellulose (HPMC, viscosity ∼50 cSt (2% (w/v) solution)), glucose, cellobiose, chitin (crab or shrimp shells), lichenan, and yeast mannan were purchased from Sigma Chemical Company. Hydroxyethylcellulose (HEC, viscosity ∼0.08−0.15 Pa (2% (w/v) solution)) was purchased from Aldrich Chemical Company. Barley β-glucan (viscosity 20−30 cSt), oat β-glucan (viscosity 10 cSt), and pachyman (lot MPA80801) were purchased from MegaZyme Ltd. (North Rocks, N.S.W., Australia). Cellooligosaccharides (cellotriose to cellohexaose, >99% pure) were from Seikagaku America Inc. Birchwood xylan (Roth 7500; MW ∼25000) was obtained from Carl Roth RG (Karlsruhe, Germany).

Protein Purification. Overnight cultures of *Escherichia coli* strain JM101, harboring pTugN1n (Johnson et al., 1996), were diluted 500-fold in tryptone−yeast extract−phosphate medium (TYP) (Sambrook et al., 1989) supplemented with 100 μg of kanamycin/mL and grown at 30 °C to an A_{600nm} of 2.0. Transcription of the gene fragment encoding CBD_{N1} was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.1−0.5 mM, and growth was continued for a further 12−18 h at 30 °C. To each liter of culture supernatant, clarified by centrifugation at 4 °C for 10 min at 13 000*g*, was added 50 g of Avicel washed in 50 mM potassium phosphate buffer, pH 7.0. After incubation overnight at 4 °C with stirring, Avicel was recovered by vacuum filtration on a Whatman GF/A or GF/C glass filter and washed with 100 mL of 1 M sodium chloride in 50 mM potassium phosphate buffer, pH 7.0, followed by 150 mL of 50 mM potassium phosphate buffer, pH 7.0. CBD_{N1} was eluted from the Avicel with distilled water. Recovery rates were increased by recombining the initial filtrate and the buffer washes with the Avicel. The above process was repeated, and the two fractions were combined, adjusted to pH 6.0 with potassium phosphate buffer (20 mM final), and further purified by anion-exchange chromatography on MacroQ (Bio-Rad). CBD_{N1} was eluted with a linear gradient of 0−1 M sodium chloride in 20 mM potassium phosphate buffer, pH 6.0. The protein was desalted, exchanged into the appropriate buffer and concentrated by ultrafiltration on a 1K filter (Filtron Technology Corp.). Approximately 85 mg of pure CBD_{N1} was obtained from 1 L of supernatant.

Titration Microcalorimetry. Isothermal titration calorimetry experiments were carried out in a Calorimetry Sciences Corp. model 4200 isothermal titration calorimeter. The ITC was calibrated daily by internal standard electrical pulses

and monthly by heat of protonation experiments in 250 mM Tris using 1 mM HCl standard. All solutions were filtered through 0.45 μm filters (Acrodisc) and thoroughly degassed by vacuum stirring prior to loading in the ITC. A 2 mL titrate solution containing 200 μM of soluble cellooligosaccharides in 50 mM potassium phosphate buffer, pH 7.0, and 0.02% sodium azide was titrated with a 2 mM solution of CBD_{N1} in the same buffer. Alternatively, cellooligosaccharides (2 mM, pH 7.0) were titrated in a 2 mL of CBD_{N1} solution. Twenty to twenty-five successive portions of 10 μL of the titrant solution were injected at 5−10 min intervals into the sample cell from a 250 μL syringe under continuous mixing at 200−250 rpm. The reference cell was filled with the same solution as the sample cell but was not titrated. Each injection generated a measurable heat resulting from the association of protein and ligand. Raw binding data were corrected for heat of dilution of both protein and sugars, obtained by independent titration experiments. The calorimetric peaks were integrated and corrected for heats of dilution, and the resulting cumulative isotherms were then regressed using a nonlinear least-squares algorithm (Wiseman et al., 1989) yielding values for the equilibrium association constant K_a (M^{-1}), the standard enthalpy change $\Delta H°$ (kJ mol^{-1}), and the stoichiometry of binding or the number of binding sites per monomer *n*. To assure accurate deconvolution of the calorimetric binding data, all experiments in the present study were performed with *c*-values (product of the association constant K_a and the total macromolecule concentration $M_t(0)$ in the titration cell) between $1 < c < 20$ (Wiseman et al., 1989). Constant pressure heat capacity changes, ΔC_p, were determined by evaluating $\Delta H°$ using isothermal titration calorimetry as a function of the temperature *T*.

Scatchard Analysis. Scatchard plots were constructed from the calorimetric data as follows. The heat evolved from the *i*th injection Q(*i*) (μJ) and the total concentration of ligand $X_t(i)$ (mM) and protein $M_t(i)$ (mM) after the *i*th injection were obtained and used to determine the concentration of bound ligand $X_b(i)$ after the *i*th injection

$$X_b(i) = \Delta X_t Q(i)/Q_{max} + X_b(i\text{-}1) \quad (1)$$

where ΔX_t is the increase in total concentration (mM) of ligand in the sample cell produced by the *i*th injection and Q_{max} (μJ) is the heat evolved for complete binding of ligand. Q_{max} is calculated from eq 2:

$$Q_{max} = X_t(1)\Delta H V_0 \quad (2)$$

where $X_t(1)$ is the total concentration of the ligand (mM) after the first injection, ΔH is the enthalpy change (J mol^{-1}), and V_0 is the cell volume (mL). The concentration of free ligand $X_f(i)$ is then given by

$$X_f(i) = X_t(i) - X_b(i) \quad (3)$$

The Scatchard plot $r(i)$ *vs* $r(i)/X_f(i)$, where $r(i) = X_b(i)/M_t(i)$, the fraction of ligand bound per mol of protein after the *i*th injection, is then prepared.

Affinity Electrophoresis. An affinity electrophoresis method using polyacrylamide gels containing ligand sugars as described by Takeo and co-workers (Mimura et al., 1992; Takeo & Kabat, 1978; Takeo & Nakamura, 1972) was developed for use in the Bio-Rad mini-gel apparatus. Separating gels contained 13% acrylamide in 1.5 M Tris·HCl buffer, pH 8.2, and stacking gels contained 3% acrylamide in the same buffer. For ligand-containing gels, glycan was added to the separating gel mixtures to 0.01−0.1% (w/v) prior to polymerization. Native polyacrylamide gels, with and without ligand, were polymerized side-by-side, separated by an internal spacer, within the same glass plates. CBD_{N1} and CBD_{N1N2} (5 μg) were electrophoresed at 4 °C and 80 V in gels with and without ligand until the tracking dye was 2 cm from the bottom of the gel. Acetylated BSA (5 μg) was used as a negative, non-interacting control. Proteins were visualized by Coomassie Blue staining.

Binding Isotherm Measurements and Competition Assays. Adsorption-isotherm measurements were carried out at 4 °C in 1.5 mL Eppendorf tubes containing CBD_{N1} or CBD_{N1N2} to a final concentration of 1−200 μM and 1 mg of crystalline cellulose (BMCC or tunicin), 2 mg of PASC or 10 mg of Avicel in 50 mM potassium phosphate buffer, pH 7.0, to a final volume of 1 mL. Control tubes contained protein without cellulose. Each solution was mixed by vortexing for 30 s and then rotated end-over-end for 2−3 h to allow the system to equilibrate. The samples were centrifuged twice for 5 min at 4 °C and 13 000*g* to remove the cellulose. Free protein left in the clarified supernatants was measured spectrophotometrically (A_{280nm}) and used to calculate the amount of CBD_{N1} or CBD_{N1N2} adsorbed to the cellulose. Each measurement was done in triplicate.

Adsorption to the β-1,4-glycans was analyzed according to the model described previously (Gilkes et al., 1992) using the relationship

$$\frac{1}{[X_b]} = \frac{1}{K_a[N_0]} \cdot \frac{1}{[X_f]} + \frac{a}{[N_0]} \quad (4)$$

where $[X_b]$ is the concentration of bound ligand (mol (g of cellulose)$^{-1}$), $[X_f]$ is the concentration of free ligand (molar), $[N_0]$ is the concentration of binding sites in the absence of ligand (mol (g of cellulose)$^{-1}$), *a* is the number of repeating units (i.e., cellobiosyl residues), and K_a is the equilibrium association constant (L mol^{-1}). The relative equilibrium constant (K_r), defined as

$$K_r = [N_0]K_a \quad (5)$$

was determined from the slope of a plot of $1/[X_b]$ *vs* $1/[X_f]$, using a double-weighted, least-squares analysis (Gilkes et al., 1992). Residuals were weighted along both axes when the sum of squares errors was minimized between the fitted line and data points. $[X_f]$ was determined from absorbance at 280 nm using the extinction coefficients of the purified materials. The concentration of bound protein $[X_b]$ was determined from the difference between the initial protein concentration and $[X_f]$.

To evaluate competing soluble glycans, binding isotherms were measured in the presence of 0.5% (w/v) HEC, barley β-glucan, CMC, or xylan. Controls containing CBD and soluble glycan but no PASC, PASC and soluble glycans, or CBD and soluble glycans were run in parallel with the analysis. K_r values were determined as described above. Equilibrium constants (K_d equivalent to IC_{50}) for binding of the soluble ligands were obtained after incubation of 2 mg

Table 1: Relative Binding Constants (K_r) for Binding of CBD_{N1}, CBD_{N1N2}, and CBD_{Cex} to Crystalline and Regenerated Cellulosic Matrices at 4 °C and pH 7.0

	K_r (L g^{-1})[a]		
ligand	CBD_{N1}	CBD_{N1N2}	CBD_{Cex} [b]
PASC	5.05 (±0.40)	9.26 (±1.20)	1.50 (±0.20)
Avicel	0.24 (±0.05)	0.35 (±0.08)	2.70 (±0.30)
BMCC	NB[c]	NB	20.8 (±0.80)

[a] Values between parentheses indicate deviation from regression analysis. K_r values were determined from the binding isotherms according to the model proposed by Gilkes et al. (1992). [b] Data taken from Ong et al. (1992) and included here for comparison. [c] NB indicates that the binding affinity was too low to detect or to measure accurately.

of PASC and 4 μM CBD in the presence of increasing amounts (0−5 mM) of the competing glucans.

Elution Experiments. Elution conditions for CBD_{N1} or CBD_{Cex} bound to cellulose were monitored by SDS−PAGE analysis. All adsorption experiments were performed in 1 mL syringes packed with 1 mg of BMCC, 2 mg of PASC or 10 mg of Avicel. After the microcolumns were washed with 5 mL of potassium phosphate buffer (50 mM, pH 7.0), samples of pure CBD_{N1} or CBD_{Cex} (0.3−0.5 mg) were added and allowed to flow freely through the column. The columns were washed with 5 mL of high-salt buffer (50 mM potassium phosphate buffer, pH 7.0, 1 M NaCl) followed by 5 mL of low-salt buffer (50 mM potassium phosphate buffer, pH 7.0), and finally distilled water, NaOH (0.5 and 1 N), HCl (0.5 and 1 N), or SDS (1 or 2%) was added as potential eluents of the bound protein. The cellulose was then transferred to a microcentrifuge tube, washed briefly with low-salt buffer, collected by centrifugation, and boiled after addition of 40 μL of SDS loading buffer. Fractions of 20 μL were then analyzed by SDS−PAGE (15%).

RESULTS AND DISCUSSION

Interaction of CBD_{N1} with Insoluble Polysaccharides

Adsorption of CBD_{N1} on insoluble cellulose is strongly dependent on cellulose structure. CBD_{N1} has highest affinity for regenerated, phosphoric acid-swollen cellulose (PASC), about 21-fold lower affinity for microcrystalline Avicel, and little or no affinity for bacterial (BMCC) or tunicin cellulose (Table 1) (Coutinho et al., 1992). Regenerated cellulose, or cellulose II, is composed of antiparallel β-1,4-glucan chains (Sarko, 1986) and is considered to be amorphous or paracrystalline (Ooshima et al., 1983). BMCC consists of parallel chains (cellulose I) in highly ordered, hydrogen bonded, crystalline microfibrils (Gilkes et al., 1992). The crystallinity of BMCC is estimated at 76% relative to Valonia, the most crystalline (100%) cellulose available (Kulshreshtha & Dweltz, 1973). Avicel, a cellulose derived from wood, is composed of various microcrystallites imbedded within an amorphous matrix. Its crystallinity is estimated to be approximately 50% (Wood, 1988). Adsorption data therefore indicate that CBD_{N1} binds amorphous and not crystalline cellulose.

The affinity of CBD_{N1N2} also decreases with increasing crystallinity of the cellulosic matrix. However, the K_r values for binding of CBD_{N1N2} to both PASC and Avicel are roughly 2-fold higher than for CBD_{N1} (Table 1). This suggests that the two repeats, CBD_{N1} and CBD_{N2}, within CBD_{N1N2} are equivalent and function independently (additively) rather than co-operatively. Calorimetric investigations are currently under way to determine this unequivocally.

In contrast to CBD_{N1} and CBD_{N1N2}, CBD_{Cex} binds all cellulose polymorphs tested, with a 13-fold greater affinity for BMCC than for PASC (Ong et al., 1993) (Table 1). The strong affinity of CBD_{Cex} for BMCC relates to the highly ordered presentation of cellobiose units on the cellulose I crystals (Ong et al., 1993). The projected surface area of the properly oriented NMR structure of CBD_{Cex} indicates that the bound protein covers *ca.* 30 cellobiose units on the 110 or $\overline{110}$ face of crystalline cellulose (Gilkes et al., 1992).

The absence of binding to BMCC points to a different binding mechanism for CBD_{N1}. Its affinity for amorphous cellulose indicates a preference for less conformationally restricted, single cellulose chains as opposed to a highly ordered array of cellulose chains. CBD_{N1} (or CBD_{N1N2}) is reversibly desorbed from Avicel by washing with either buffer or distilled water. This differs from many other CBDs characterized to date, which can often only be eluted from Avicel under harsher conditions involving addition of guanidinium hydrochloride (>3.5 M) (Ong et al., 1993), SDS (Millward-Sadler et al., 1994), or triethylamine (1%) (Goldstein et al., 1993).

Effect of pH and Salts on Binding of CBD_{N1} to PASC

Most CBDs, such as CBD_{Cex} from *C. fimi* and CBD_{CBHI} from *T. reesei*, are characterized by low charge densities due to the low number of both acidic and basic amino acid residues (Gilkes et al., 1991; Tomme et al., 1995b). As a result, the affinities of these CBDs for their insoluble glucans are relatively insensitive to pH and salt concentrations (Creagh et al., 1996; Kyriacou et al., 1988; Ong et al., 1993), although high concentrations of multivalent cations have been shown to influence binding (Reinikainen et al., 1995).

CBD_{N1} is somewhat atypical in that it contains three basic (Arg) and 21 acidic (Glu and Asp) residues. This not only increases its net charge but also makes the protein considerably more acidic (calculated pI = 3.5) than most CBDs. The influence of pH and salt concentration on the binding of CBD_{N1} on PASC were therefore investigated. Binding is strongest at pH 5.0 (K_r = 5.6 ± 0.4 L g^{-1}) and decreases slightly with increasing pH between 5.0 and 9.0; the relative affinity constants are 5.1 (±0.1) and 4.0 (±0.5) at pH 7.0 and 9.0, respectively. This correlates directly with the catalytic activity of CenC, which is optimal near pH 5.0 (Tomme et al., 1996). At high pH, the binding affinity of CBD_{N1} drops 3−4-fold (K_r = 1.7 ± 0.1 L g^{-1} at pH 11). Below pH 5.0, CBD_{N1} precipitates out of solution and binding to PASC could not be quantified. These results differ from those for binding of CBD_{Cex} to BMCC which display near-constant K_r values between pH 3.0 and 11 (Ong et al., 1993).

The influence of salt composition was investigated by binding CBD_{N1} to PASC at pH 7.0 in the presence of 1 M NaCl, 1 M $CaCl_2$, or 1 M $MgSO_4$. Potassium phosphate is known to complex multivalent cations like Ca^{2+}. Tris·HCl buffer (50 mM, pH 7.0) was therefore used in these experiments. In the absence of added salts, both buffers yield similar K_r values (5.2 ± 0.4 L g^{-1}) for binding of CBD_{N1} to PASC. Addition of 1 M NaCl does not effect the binding constant. However, bivalent ions modestly increase the

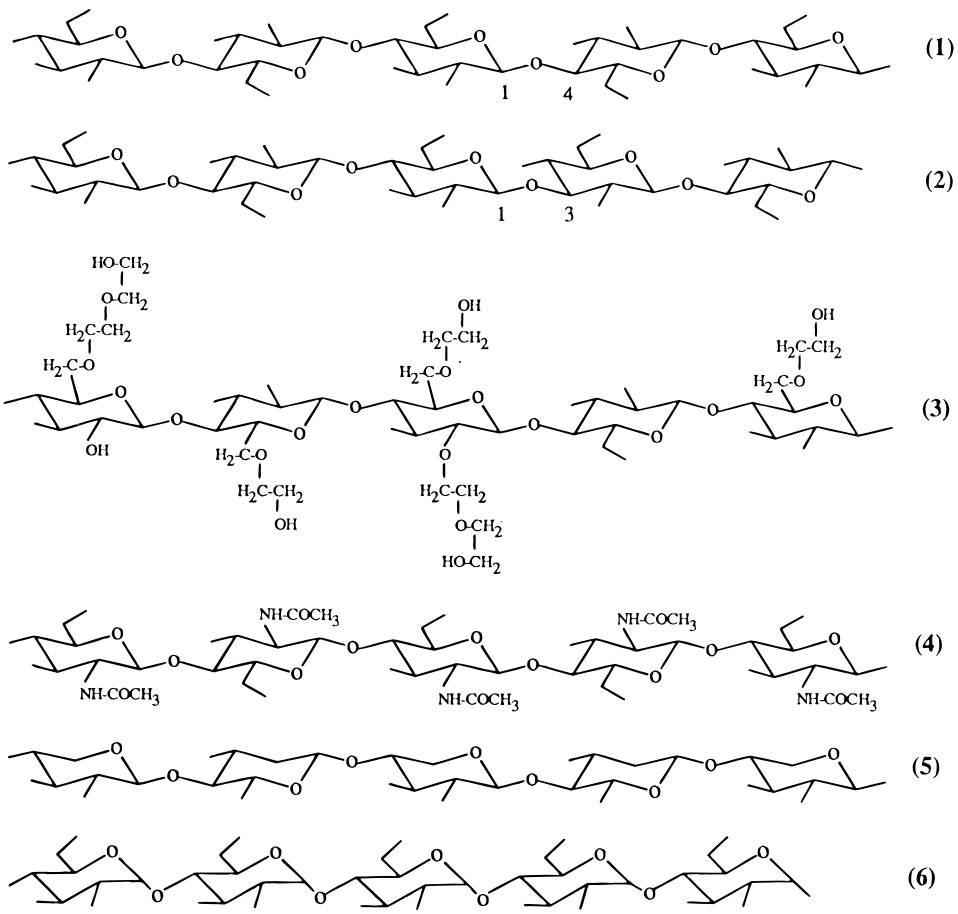

FIGURE 1: Structures of the glycans used for screening and characterization of the CBD_{N1}−carbohydrate binding. (1) β-1,4-Cellooligosaccharide (cellopentaose) chain as found in cellulose; (2) β-1,3-1,4-glucan as found in barley and oat glucan; (3) hydroxyethyl cellulose; (4) *N*-acetylglucosamine chain as found in chitin; (5) β-1,4-xylooligosaccharide backbone of xylan; and (6) α-1,4-maltooligosaccharide found in starch.

affinity of CBD_{N1} for PASC. K_r values are 7.6 (±0.2) and 8.9 (±0.7) L g^{-1} in the presence of 1 M $MgSO_4$ and 1 M $CaCl_2$, respectively, which represent 36% and 59% increases in affinity, respectively. This increase in binding affinity is not an artifact of "salting-out" or precipitation of CBD_{N1} since the protein concentration in the controls without cellulose did not change during the incubation. Although the presence of a Ca^{2+} or a Mg^{2+} binding site in CBD_{N1} cannot be ruled out, a more probable explanation for the influence of bivalent cations is their ability to more effectively neutralize intramolecular electrostatic repulsion of negative charges on the protein surface, and possibly near the active site, due to the presence of numerous acidic residues in close proximity. Overall, however, electrostatic forces appear to make little contribution to the driving force for CBD_{N1} binding.

Carbohydrate Binding Specificity of CBD_{N1}

The fact that CBD_{N1} prefers to bind to cellulose chains with a relatively high degree of conformational freedom (e.g., in amorphous cellulose) suggests that the protein may also bind to soluble glycan polymers. CBD_{N1} was therefore screened for carbohydrate-binding activity against high molecular weight, soluble glycans by affinity electrophoresis (AE) (Figures 1 and 2; Brummel et al., 1993; Mimura et al., 1992; Takeo & Kabat, 1978; Takeo & Nakamura, 1972). AE allows for an easy, rapid and sensitive determination of the carbohydrate-binding specificities of CBD_{N1} and CBD_{N1N2}. Although AE can be used for the measurement of binding constants (Mimura et al., 1992), it was only used in this work as a screen for binding specificity (Table 2).

Figure 2 shows a typical gel for two interacting glucans (HEC and barley β-glucan) and a non-interacting carbohydrate control (xylan). In the absence of ligand, the negatively charged CBD_{N1} and CBD_{N1N2} migrate over a considerable distance in the gels under native conditions at pH 8.2 (Figure 2). However, the strong interaction with HEC or β-glucan severely decreases the mobility of CBD_{N1}, which remains at the top of both gels (Figure 2A and 2B). As demonstrated with the xylan control (Figure 2C), retardation of CBD_{N1} is not caused by viscosity or other nonspecific changes in the gel due to the presence of the glycans. Moreover, BSA is not retarded in the gels, indicating that binding between the CBDs and HEC or β-glucan is specific. Several other glycans were tested by this method (Figure 1) and the results, summarized in Table 2, show that binding of CBD_{N1} is restricted to β-1,4-linked glucans. CBD_{N1} did not bind to glucose, cellobiose, mannan, xylan, arabinogalactan, starch (amylose or amylopectin), dextran T70, laminarin, or pachyman. For several of these glycans, AE results were verified by other techniques including NMR (Johnson et al., 1996), competition assays, and calorimetry.

Binding Thermodynamics for Soluble Oligosaccharides

The energetics of the interaction of CBD_{N1} with various high and low molecular weight, soluble β-1,4-glucans were

Table 2: Semiquantitative Evaluation of the CBD_{N1} Carbohydrate-Binding Specificity As Determined by Various Methods

ligand	binding[a]	detection method[b]
soluble ligands		
cellotriose	+/−	NMR/ITC
cellotetraose	++	NMR/ITC
cellopentaose	+++	NMR/ITC
cellohexaose	+++	NMR/ITC
carboxymethylcellulose (CMC)	+	AE/competition assay
hydroxyethylcellulose (HEC)	+++	AE/competition assay
hydroxypropylmethylcellulose (HPMC)	+++	AE
barley β-glucan	+++	ITC/AE/competition assay
oat β-glucan	+++	ITC/AE
chitosan	+/−	AE
glucomannan	+	AE
insoluble ligands		
phosphoric acid-swollen cellulose (PASC)	+++	binding isotherms
Avicel	+	binding isotherms
chitin	+/−	binding isotherms
Sephadex	+/−	binding isotherms

[a] Semiquantitative measurement: (+/−) weak binding, (+) clearly observable affinity, (++) strong, specific interaction, (+++) very strong binding. *b*Detection method: NMR, nuclear magnetic resonance; ITC, isothermal titration calorimetry; AE, affinity electrophoresis.

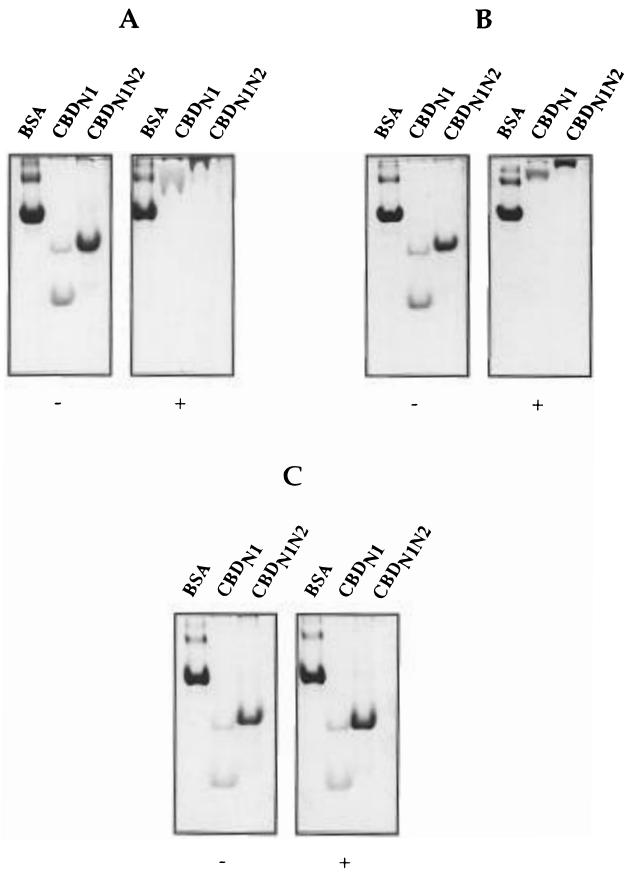

FIGURE 2: Affinity electrophoresis of CBD_{N1} and CBD_{N1N2} in the absence (−) and presence (+) of barley β-glucan (0.1% w/v) (A), hydroxyethylcellulose (0.1% w/v) (B), or birch wood xylan (0.1% w/v) (C).

studied in detail by isothermal titration calorimetry. Figure 3 shows the experimental cumulative calorimetric heat released in the binding of CBD_{N1} to cellopentaose in 50 mM phosphate buffer, pH 7.0 at 35 °C. The cumulative heats for titration of CBD_{N1} into cellopentaose or cellopentaose into CBD_{N1} are identical, indicating a 1:1 stoichiometry. Binding is also completely reversible (data not shown). The nonlinear least-squares fit of the cumulative heat data assuming a 1:1 equilibrium model is also shown in Figure

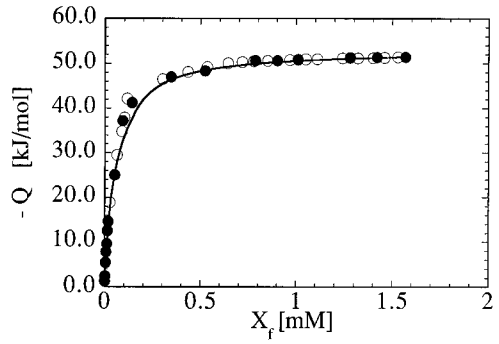

FIGURE 3: Experimental cumulative heat for binding, after correction for the heat of dilution, released during titration of cellopentaose with CBD_{N1} (●) or titration of CBD_{N1} with cellopentaose (○) at 35 °C in 50 mM potassium phosphate buffer, pH 7.0. The solid line represents the least-squares Langmuir model fit to the integrated data assuming a 1:1 stoichiometry.

3. Binding of cellopentaose to CBD_{N1} is exothermic, with a standard enthalpy of binding $\Delta H°$ of -53.0 ± 1.3 kJ mol^{-1}. The thermodynamic parameters for interaction of CBD_{N1} with various β-1,4-linked oligosaccharides at pH 7.0 and 35 °C are summarized in Table 3.

Figure 4 shows the Scatchard plot derived from calorimetric data for binding of cellopentaose to CBD_{N1}. The linear result indicates that there is only one class of binding sites and only one binding site per monomer. The stoichiometry for binding to CBD_{N1} is also 1:1 for all other small oligosaccharides; *n*-values regressed from calorimetry data are between 0.86 and 0.95 (Table 3). This is consistent with the ultracentrifugation studies that demonstrate that CBD_{N1} is a monomeric protein and with NMR studies which identified a single binding site (Johnson et al., 1996). In contrast, starch-binding domains from the glucoamylase from *Aspergillus niger*, although somewhat similar to CBD_{N1} in that they bind soluble and insoluble α-1,4 linked glucose polymers, have two binding sites and bind soluble maltosaccharides with a 2:1 (ligand/protein) stoichiometry (Sigurskjold et al., 1994).

The larger polymers, oat β-glucan (DP = 45), barley β-glucan (DP = 93), and hydroxyethylcellulose (DP ∼ 400 glucose units), bind on average three to four CBD_{N1}

Table 3: Thermodynamic Parameters for Binding of CBD_{N1} to Various Glucans at 35 °C and pH 7.0 As Determined by Isothermal Titration Calorimetry

carbohydrate	K_a (M^{-1})	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1})	ΔC_p [b] (J mol^{-1} K^{-1})	n
cellotetraose	$3.2 (\pm 0.5) \times 10^3$	-20.7 (± 3.5)	-40.4 (± 1.7)	-19.7 (± 3.8)	ND[c]	0.92
cellopentaose	$2.1 (\pm 0.3) \times 10^4$	-25.5 (± 2.7)	-53.0 (± 1.3)	-27.6 (± 3.5)	-209.2 (± 33.5)	0.86
cellohexaose	$2.2 (\pm 0.4) \times 10^4$	-25.6 (± 2.3)	-54.4 (± 1.3)	-28.8 (± 3.1)	-259.4 (± 37.7)	0.95
barley β-glucan	$2.9 (\pm 0.4) \times 10^4$	-26.3 (± 2.7)	-57.4 (± 2.1)	-31.1 (± 3.3)	ND	3.81
oat β-glucan	$2.5 (\pm 0.4) \times 10^4$	-25.9 (± 3.1)	-60.2 (± 0.6)	-34.3 (± 4.0)	ND	3.62
hydroxyethyl cellulose	$2.2 (\pm 0.4) \times 10^4$	-25.6 (± 2.3)	-56.2 (± 2.1)	-30.6 (± 3.5)	-431.0 (± 33.5)	3.16
PASC	$1.5 (\pm 0.4) \times 10^4$	-24.5 (± 3.3)	-32.1 (± 3.4)	-7.6 (± 2.4)	ND	ND

[a] Values in parentheses indicate the standard deviation of fit between the experimental binding curve and the calculated curve obtained with the fitted parameters or three standard deviations from regression analysis. [b] Calculated as $\Delta\Delta H°/\Delta T$ from the binding enthalpy values determined at 25 and 35°C. [c] ND indicates that the values were not determined.

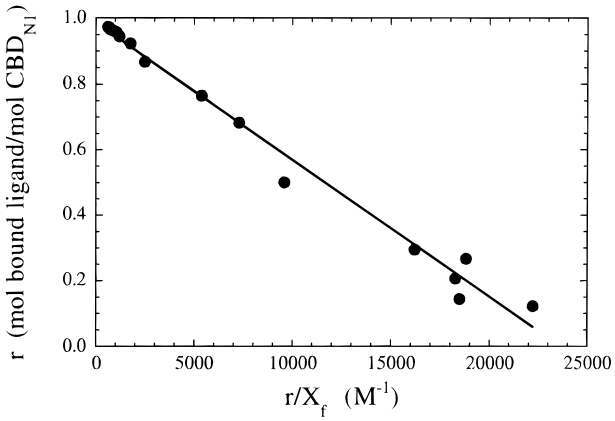

FIGURE 4: Scatchard plot for binding of cellopentaose to CBD_{N1} at 35 °C in 50 mM potassium phosphate, pH 7.0. The values for Q_{max} was calculated according to eq 2 in Materials and Methods. The stoichiometry for binding ($n = 0.96$) is the ordinate intercept when $r(i)/X_f(i) = 0$. The association constant $K_a = 21\,800 \pm 400$ M^{-1} was determined from the slope of the line.

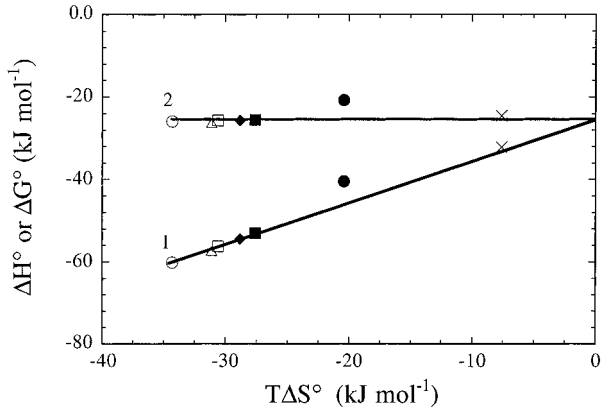

FIGURE 5: Enthalpy−entropy compensation plots for the binding of cellotetraose (●), cellopentaose (■), cellohexaose (◆), barley β-glucan (△), oat β-glucan (○), HEC (□), and PASC (×) to CBD_{N1} at pH 7.0 and 35 °C. The straight line for $\Delta H°$ (line 1) was obtained by linear regression and has a slope of 1.06 with an intercept of -24.0 kJ mol^{-1} ($r = 0.999$). The straight line for $\Delta G°$ (line 2) was also obtained by linear regression and has a slope of 0.06 and an intercept of -24.1 kJ mol^{-1} ($r = 0.912$).

molecules per polymer (Table 3). All thermodynamic values in Table 3 are expressed per mole of CBD_{N1}, i.e., per mole of binding site.

For all soluble sugar ligands, binding to CBD_{N1} is controlled by a favorable standard enthalpy change compensated by a significant decrease in entropy; i.e., the signs for both $\Delta H°$ and $\Delta S°$ are negative, and the magnitude of the binding enthalpy exceeds that of the entropy (Table 3). This is typical for soluble carbohydrate−protein interactions (Chervenak & Toone, 1995; Lemieux, 1989; Munske et al., 1984), which are almost always enthalpically driven (Quiocho, 1986). Unfavorable entropy contributions to binding (Carver et al., 1989; Kronis & Carver, 1985) and enthalpy−entropy compensation (Herron et al., 1986; Hindsgaul et al., 1985; Sigurskjold & Bundle, 1992) are also common in protein−sugar interactions.

The standard free energy $\Delta G°$ and enthalpy $\Delta H°$ changes are essentially invariant with chain length for glucans with a DP ≥ 5, suggesting that five to maximum six glucopyranosides fill the binding site. This is in agreement with structural data which shows that the carbohydrate-binding cleft in CBD_{N1} extends over about five glucose residues (Johnson et al., 1996). Larger chains extend out of the binding cleft without further contributing to the binding, although some favorable interactions at the periphery of the binding site may occur. In contrast, decreasing the chain length of cellopentaose by one glucose unit decreases the association constant 6−7-fold. The thermodynamic contributions to binding of the extra glucose residue in the pentaoside *vs* the tetraoside are estimated to be $\Delta\Delta G° = -4.8 \pm 0.4$, $\Delta\Delta H° = -12.6 \pm 0.5$, and $T\Delta\Delta S° = -7.2 \pm 0.8$ kJ mol^{-1}. These values are close to the average $\Delta H°$ and $T\Delta S°$ values per glucose residue which, for both the tetraoside and the pentaoside, are -10.3 ± 0.3 and -5.3 ± 0.2 kJ mol^{-1}, respectively. The relatively large exothermal change in enthalpy upon binding suggests the involvement of van der Waals forces between the extended site and the glucan chain in addition to strong intermolecular hydrogen bond formation. For instance, van der Waals interactions may arise from stacking of the fifth glucopyranoside moiety against a tyrosine residue, probably Tyr19, as observed from the perturbation of the protein UV difference spectra induced upon binding of cellopentaose or hexaose (Johnson et al., 1996).

Enthalpy−Entropy Compensation

If a group of analogous ligands bind by the same mechanism, then a linear relationship between enthalpy and entropy can be expected (Carver et al., 1989; Leffer & Grunwald, 1963). This relationship defines the extent of the energy compensation. Figure 5 shows the enthalpy−entropy compensation plot ($-\Delta H°$ versus $-T\Delta S°$) for glucan binding to CBD_{N1}. For all of the ligands studied, the favorable change in enthalpy is compensated by a negative change in entropy which is thought to be due to the freezing out of a single conformer from multiple conformational microstates available to the unbound polysaccharide and the protein

Table 4: Influence of Salt Concentration on Binding of CBD_{N1} to Cellopentaose at 35 °C and pH 7.0 As Determined by Isothermal Titration Calorimetry

solvent	K_a (M^{-1})	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$T\Delta S°$ (kJ mol^{-1})	$\Delta Cp°$ (J mol^{-1} K^{-1})	n
pure water (pH 7.0)	5.2 (±0.7) × 10^3	−21.9 (±3.8)	−43.2 (±2.6)	−21.3 (±4.0)	ND	0.69
50 mM potassium phosphate, pH 7.0	2.1 (±0.3) × 10^4	−25.5 (±2.7)	−53.0 (±1.3)	−27.6 (±3.5)	−209.2 (±33.5)	0.86
50 mM potassium phosphate, pH 7.0 + 1 M NaCl	2.6 (±0.3) × 10^4	−26.0 (±2.4)	−56.8 (±1.5)	−30.4 (±2.8)	−401.7 (±33.6)	0.96

(Carver et al., 1989). Binding therefore restricts the translational, rotational, and conformational degrees of freedom of both the sugar polymer and the side chains in the binding site of the protein. Additional components of the enthalpy−entropy compensations are due to the unique properties of water and the displacement of water molecules during formation of the bound complex (Lemieux, 1989; Privalov & Makhatadze, 1993). Tight binding of a soluble oligosaccharide requires a loss in conformational entropy for that portion of the polymer chain in direct contact with the protein binding site, which compensates the entropy gain associated with dehydration of the protein−ligand contact surface. Both configurational and solvation entropies typically scale roughly with ligand size, presumably a reflection of both the number of solvent molecules stripped and the number of freely rotating bonds restricted during binding (Chervenak & Toone, 1995). However, in protein-soluble carbohydrate interactions, solvation effects make a relatively small contribution to the enthalpy change upon binding (Chervenak & Toone, 1995), and they do not appear to make a substantial contribution to the standard entropy change, which is dominated by losses in ligand (and protein) configurational entropy (Chervenak & Toone, 1995; Kelley & O'Connell, 1993).

The slope of the compensation plot can be directly related to the specific heat capacity change, ΔC_p, of binding (Sigurskjold & Bundle, 1992). This in turn is determined by the net balance of polar and nonpolar interactions. The slope of the compensation curve shown in Figure 5 is 1.06. A slope greater than 1 suggests a binding interaction involving mainly polar interactions (Sigurskjold et al., 1994). This result is consistent with the predominance of planar polar and charged residues in the carbohydrate-binding site of CBD_{N1} (Johnson et al., 1996) and with the small heat capacity change associated with the binding event (see below). The ordinate intercept of the compensation plot is -24.0 ± 1.5 kJ mol^{-1}, which is close the mean free energy change $\Delta G°$ of -25.8 ± 2.5 kJ mol^{-1} for the six ligands and those observed for a number of carbohydrate−protein systems (Hindsgaul et al., 1985; Lemieux, 1989).

It should be clear from Figure 5 that binding of CBD_{N1} to PASC follows the same mechanism as for the soluble glycans; the data point for PASC falls on the same straight line as the other polymers. This confirms that the driving forces for binding of CBD_{N1} to soluble glucans and to PASC are the same but are distinctively different than those for binding of CBD_{Cex} to BMCC (Creagh et al., 1996).

Heat Capacity of Binding of Cellopentaose, Cellohexaose, and Hydroxyethylcellulose

Calorimetric determinations at two different temperatures (25 and 35 °C) provided an estimate of ΔC_p, the heat capacity changes of binding for cellopentaose, cellohexaose, and hydroxyethylcellulose. The regressed ΔC_p values for each system are −209.2 (±33.5), −259.4 (±37.7), and −431 (±33.5) J K^{-1} mol^{-1}, respectively (Table 3). These values of ΔC_p are all negative and relatively small in comparison with a wide variety of other binding phenomena involving proteins (Sturtevant, 1977). Murphy and Gill (1990) have suggested that ΔC_p is directly proportional to the change in polar (pol) and apolar (apol) solvent accessible surface area ΔA

$$\Delta C_p = \Delta A_{pol}\Delta C°_{p\,pol} + \Delta A_{apol}\Delta C°_{p\,apol} \qquad (6)$$

The small ΔC_p values observed here suggest (1) that hydrogen bonding and other polar interactions are balanced by apolar interactions in the CBD_{N1}-glucan binding event and (2) that binding is not dominated by the hydrophobic effect. For binding processes dominated by dehydration events, i.e., burying of apolar groups away from the aqueous solvent with displacement of water molecules upon complex formation, ΔC_p is large and negative (Livingstone et al., 1991; Spolar & Record, 1994; Sturtevant, 1977). Changes in ionization and significant conformational changes, including low-frequency vibrational modes (Sturtevant, 1977) upon molecular association can also be excluded since these effects are also associated with much larger changes in specific heat capacity (Bains et al., 1992).

Contribution of Electrostatic Effects

The influence of salt concentration on the binding energetics of CBD_{N1} to cellopentaose is summarized in Table 4. A 4-fold higher K_a is observed for binding in 50 mM phosphate buffer relative to that in water, indicating that electrostatic interactions oppose binding of soluble sugars. An increase in ionic strength can influence electrostatic interactions in three general ways: (1) by reducing intermolecular charge−charge interactions (e.g., protein−protein or protein−polymer interactions), (2) by sequestering free water molecules and thereby reducing the fraction of free water available to solvate the protein and ligand, and (3) by reducing intramolecular charge−charge interactions between proximal charged groups on the surface of the protein or polymer. For the 1 mM CBD_{N1} solution at pH 7.0 used in this study, the Debye length characterizing electrostatic interactions is *ca.* 8 Å in pure water, 5.4 Å in 50 mM phosphate buffer, and 2.7 Å in 50 mM phosphate buffer containing 1 M NaCl. The average distance between macromolecules in this solution is *ca.* 75 Å suggesting that intermolecular electrostatic interactions (process 1) are not important.

On the basis of ion-hydration numbers (Robinson & Stokes, 1970), 50 mM phosphate buffer and 1 M NaCl bind approximately 1.5% and 15% of the total water in the solution, respectively. However, within experimental error, addition of 1 M NaCl to the phosphate buffer does not significantly affect the thermodynamics of binding of CBD_{N1} with the sugar. This result is in agreement with the small

ΔC_p values shown in Table 4 and indicates that dehydration effects (process 2) are not responsible for the observed ionic strength dependence. We therefore conclude that reduced intramolecular electrostatic repulsions between the carboxylates of acidic residues in closer proximity than *ca.* 5.0 Å (process 3) are most likely responsible for the increased affinity of CBD_{N1} for cellopentaose in phosphate buffer.

Comparison with Other Carbohydrate- and Cellulose-Binding Proteins

Although sugar-binding proteins differ remarkably in their overall tertiary structures, CBD_{N1} shares similar general features with other carbohydrate-binding proteins such as lectins, carbohydrate-specific antibodies, and starch-binding domains. Many of these proteins have a somewhat shallow carbohydrate binding groove near the surface of the protein. In the binding site, planar polar residues with two or more functional groups (i.e., Asp, Asn, Glu, Gln, His, and Arg) as well as Lys residues, are frequently found. These residues are thought to be involved in the specific interaction with carbohydrates through extensive hydrogen-bonding networks to sugar hydroxyls thereby fixing the essential residues and solvent molecules in a favorable orientation for maximum sugar-binding interactions (Quiocho, 1988, 1989). As a result, binding is largely driven and controlled by the accompanying favorable change in standard enthalpy. Van der Waals interactions, especially stacking type van der Waals forces between the sugar and aromatic amino acid residues in the binding pocket, also contribute significantly to this enthalpic driving force and confer both stability and specificity to the protein−sugar complex (Vyas, 1991). A relatively large number of tryptophan and tyrosine residues are usually present in the binding sites of sugar-binding and polysaccharide-degrading proteins. CBD_{N1} is somewhat different in that the carbohydrate-binding channel is almost devoid of aromatic residues (Johnson et al., 1996).

Although interaction between β-1,4-glucanases and their (insoluble) polysaccharide substrates continues to be the subject of intense study (Tomme et al., 1995a), few thermodynamic data are available for these interactions. As a result the energetics and mechanism of binding of the individual cellulose-binding domains to cellulose are not well understood. Only the binding mechanism of CBD_{Cex} on BMCC is reasonably well characterized (Creagh et al., 1996). The cellulose contact surface of CBD_{Cex} has a relatively hydrophobic binding ridge containing three linearly aligned and solvent-exposed tryptophan residues (Xu et al., 1995) involved in binding to cellulose (Din et al., 1994). Binding is driven by a relatively large change in entropy resulting from significant dehydration of the protein and ligand contact surfaces (Creagh et al., 1996).

The difference in mechanism of binding for CBD_{N1} to PASC or soluble glucans compared with CBD_{Cex} to BMCC (Creagh et al., 1996) can, at least in part, be attributed to the nature of the cellulose ligand. The conformation of each cellulose chain on the fiber surface of BMCC is essentially fixed, and the chains are unlikely to undergo any loss in conformational entropy upon binding. In contrast, a soluble cellulose or glucan−polymer chain, which in solution can sample a large number of conformational states, will undergo a substantial loss in conformational entropy upon binding, which is largely uncompensated due to the relatively small accompanying changes in solvational entropy. Although the structure of PASC is ill defined, our results suggest that at least a portion of the cellulose chains in PASC have a high conformational degree of freedom similar to the β-glucan or HEC chains in solution. Thus, the calorimetric study of binding of CBDs to various cellulosic matrices may ultimately lead to valuable and much needed insights into the nature of the substrates and to their influence on the catalytic activities and efficiencies of the cellulases and/or xylanases.

Role of CBD_{N1} in Cellulose Degradation

Cellulose-binding domains play various roles in the degradation of cellulose, the most obvious of which is to increase the local enzyme concentration at the cellulose surface by allowing the cellulases to interact more tightly and closely with the substrate (Gilkes et al., 1991; Knowles et al., 1988). Hydrolysis of soluble sugars is not enhanced by and indeed does not require the presence of a CBD (Gilkes et al., 1988; Tomme et al., 1988). The occurrence of a CBD which binds soluble β-1,4- (or β-1,3-1,4-) linked oligomers of glucose in an enzyme (β-1,4-glucanase CenC) known to act on insoluble cellulose (Tomme et al., 1996) is therefore somewhat puzzling. The same holds true for glucoamylases (e.g., glucoamylase 1 from *A. niger*) which hydrolyze insoluble granular starch, but not soluble maltodextrins, with increased rates due to the presence of a starch-binding domain (Svensson et al., 1982; Takahashi et al., 1985). Several hypotheses can be proposed for the binding specificity of CBD_{N1}. (1) CBD_{N1} has truly evolved to bind cellulose chains within the amorphous regions of cellulose, and binding of soluble glucans is coincidental due to the similar presentation of the chains. (2) CBD_{N1} captures and solubilizes cellooligosacharides released by the action of endoglucanases on cellulose. This would prevent these cellooligosaccharides, which show a strong tendency to aggregate together or to associate with the cellulose surface when their DP $\geq$ 4, from being wasted due to the inaccessibility of the aggregates for the endo- and exoglucanase. Finally, (3) CenC is somehow involved in the induction of the cellulase cascade; soluble glucans, signaling the presence of cellulose, are hydrolyzed by CenC to cellobiose (Tomme et al., 1996), a known inducer of cellulases (Bhat et al., 1993; Kubicek, 1993), and/or are captured by CBD_{N1} and internalized in *C. fimi* cells. CenC is rarely detected in the *C. fimi* growth medium (Sandercock et al., 1996), and the possibility therefore exists that CenC is associated with the cell wall, possibly mediated through the C-terminal repeats C1C2. CenC would then have no obvious need to bind amorphous cellulose, unless the enzyme is released from the cell wall after induction and/or exhaustion of the initial glucans.

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