The interaction of carbohydrate-binding modules with insoluble non-crystalline cellulose is enthalpically driven

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Natural cellulose exists as a composite of cellulose forms, which can be broadly characterized as crystalline or non-crystalline. The recognition of both of these forms of cellulose by the CBMs (carbohydrate-binding modules) of microbial glycoside hydrolases is important for the efficient natural and biotechnological conversion of cellulosic biomass. The category of CBM that binds insoluble non-crystalline cellulose does so with an affinity approx. 10–20-fold greater than their affinity for cello-oligosaccharides and/or soluble polysaccharides. This phenomenon has been assumed to originate from the effects of changes in configurational entropy upon binding. The loss of configurational entropy is thought to be less profound upon binding to conformationally restrained insoluble non-crystalline cellulose, resulting in larger free energies of binding. However, using isothermal titration calorimetry, it is shown that this is not the case for the high-affinity interactions of CcCBM17 (the family 17 CBM from EngF of *Clostridium cellulovorans*) and BspCBM28 (the family 28 CBM from Cel5A of *Bacillus* species 1139) with regenerated cellulose, an insoluble preparation of primarily non-crystalline cellulose. The enhanced free energy of binding of non-crystalline cellulose relative to cello-oligosaccharides is by virtue of improved enthalpy, not entropy.

Key words: adsorption, carbohydrate-binding module, cellulose, cellulose-binding domain, non-crystalline, thermodynamics.

INTRODUCTION

CBMs (carbohydrate-binding modules) are discrete modules found within numerous carbohydrate-active enzymes. Their near ubiquity in plant cell wall polysaccharide-degrading enzymes is testament to their importance in the recycling of this abundant carbon source. CBMs that are specific for insoluble cellulose, arguably the most predominant CBMs, can be grouped into two general categories: those that interact with crystalline cellulose and those that interact with non-crystalline cellulose. The former, which fall into the Type A CBMs [1], make up a unique class of carbohydrate-binding proteins that have been the subject of a number of studies over the last 15 years. The latter category of CBMs is included in Type B [1], and has only recently been gaining more attention.

Type B CBMs have binding sites that are 'valley'-shaped and well suited to accommodating individual glycan chains of three sugars or longer [1]. Those Type B CBMs that are specific for cellulose (e.g. CfCBM4-1 [2], CfCBM4-2 [3], CcCBM17 [4,5] and BspCBM28 [6]) bind soluble cello-oligosaccharides in addition to insoluble cellulose. In general, the association constants of these CBMs for their optimal cello-oligosaccharide ligands and/or soluble polysaccharides are $\sim 2 \times 10^4\ \mathrm{M}^{-1}$. The thermodynamics of these interactions are typified by large favourable ΔH (change in enthalpy) values and partially offsetting unfavourable $T\Delta S$ values, like those of most other protein–carbohydrate interactions. Interestingly, the association constants (K_a values) of these CBMs for non-crystalline insoluble cellulose are invariably greater than $10^5\ \mathrm{M}^{-1}$. Recent studies of the adsorption of CcCBM17 (the family 17 CBM from EngF of *Clostridium cellulovorans*) and BspCBM28 (the family 28 CBM from Cel5A of *Bacillus* species 1139) showed that a preparation of RC (regenerated cellulose), a primarily non-crystalline cellulose in an insoluble form, presented

at least two classes of binding sites to each of these CBMs [7]. The high-affinity sites were bound with K_as upwards of $\sim 10^6\ \mathrm{M}^{-1}$, and the low-affinity sites with K_as of $\sim 10^4\ \mathrm{M}^{-1}$. An important question is how these Type B CBMs interact with insoluble non-crystalline cellulose 10–100-fold more tightly than with sugars free in solution. This question is particularly salient considering that natural cellulose, the true ligand for these CBMs, is insoluble.

ITC (isothermal titration calorimetry) is a powerful method for studying molecular interactions. A single experiment can be used to determine the K_a, ΔH and stoichiometry (n) of an interaction. From these values, ΔG (free energy of binding) and ΔS (change in entropy) can be derived. This method has been applied twice to the challenging problem of studying the interaction of CBMs with insoluble cellulose. The binding of the family 9 CBM from *Thermotoga maritima* to RC revealed an enthalpically favourable binding mechanism. However, this CBM is the only one to be shown to interact with the reducing termini of polysaccharides, making this a somewhat unique situation [8]. Creagh et al. [9] used ITC to quantify the interaction of CfCBM2a, a family 2 CBM from *Cellulomonas fimi*, with a highly crystalline form of cellulose, BMCC (bacterial microcrystalline cellulose). This seminal study showed that, in contrast with most protein–carbohydrate interactions, this process was driven by a favourable ΔS. This thermodynamic signature was rationalized to result from the release of ordered water from the relatively apolar CBM binding site and cellulose surface. In many systems, favourable contributions to ΔS from such dehydration may be wiped out by compensating losses in ΔS from changes in the configurational entropy of the reactants. However, in the case of this CBM, the ligand (i.e. cellulose) has a rigid and fixed structure. Thus it was argued that the favourable ΔS of the CBM2a–BMCC interaction results from the lack of the loss of configurational entropy in BMCC upon binding. This assumption has been applied to

the interaction of Type B CBMs with non-crystalline cellulose, such as RC or acid-swollen cellulose, to rationalize the differences in affinities between non-crystalline cellulose and cello-oligosaccharides. Due to its insoluble nature and somewhat restrained structure, non-crystalline cellulose is thought to lose less configurational entropy than a cello-oligosaccharide upon binding, resulting in a smaller entropic penalty and, thus, better overall free energy of binding. This supposition is logical, but currently not supported by experimental data. In order to examine this, ITC was used to measure the thermodynamics of the binding of *Cc*CBM17 and *Bsp*CBM28 to RC. The results confirm the presence of two classes of binding sites for each CBM on RC. The argument of less net loss of configurational entropy may apply to the recognition of the low-affinity site, but this is offset by a less favourable ΔH of binding. Recognition of the high-affinity site at the temperatures used (15–35 °C) is a distinctly enthalpically driven process whereby the enhanced ΔG of binding relative to cello-oligosaccharides comes by virtue of improved enthalpy, not entropy.

MATERIALS AND METHODS

Materials

Microcrystalline cellulose (Avicel™ PH101) was obtained from FMC International (Little Island, County Cork, Ireland). RC was prepared by phosphoric acid treatment of Avicel™ as reported previously [8]. The batch of RC used in this study was the same as that used for previous studies of *Cc*CBM17 and *Bsp*CBM28 [7].

Purification of CBMs

Gene fragments encoding CBMs were cloned, expressed and the products purified as described elsewhere [5,6]. The CBMs used in this study were *Cc*CBM17, the C-terminal module from *Clostridium cellulovorans* Cel5A, and *Bsp*CBM28, the C-terminal module from *Bacillus* sp. 1139 Cel5A. The concentrations of purified proteins were determined by UV absorbance (280 nm) using calculated molar absorption coefficients [10] of $31\,010\,M^{-1} \cdot cm^{-1}$ and $32\,290\,M^{-1} \cdot cm^{-1}$ for *Cc*CBM17 and *Bsp*CBM28 respectively.

ITC analysis

All ITC experiments were carried out with a VP-ITC isothermal titration calorimeter (MicroCal Inc., Northampton, MA, U.S.A.). *Cc*CBM17, *Bsp*CBM28 and RC were dialysed together and extensively (3 weeks with buffer changes every 2–3 days; last change was left for 4 days) against 50 mM potassium phosphate buffer, pH 7.0. The concentrations of *Cc*CBM17 and *Bsp*CBM28, filtered previously through 0.2 μm filters, were determined to be 1.202 mM and 1.14 mM respectively, by UV absorbance. Appropriate dilutions of the proteins for the UV readings were prepared by mass on an analytical balance, assuming a density of 1 g/ml for the protein solution. The concentration of RC was determined by dry weight and corrected for the mass of buffer components, which were also measured by the dry weight of residue in the dialysis buffer. The stock of RC was diluted to 5 mg/ml in the dialysis buffer and the concentration reconfirmed by dry weight. Protein and cellulose samples were degassed extensively with stirring prior to use. Titrations were performed by titrating 26 samples of 10 μl of protein into the ITC cell, which contained RC at 5 mg/ml. The sample was stirred at 550 rev./min during the titration. Triplicate titrations were performed at each of the temperatures 15, 25 and 35 °C. Titration of buffer into buffer and

buffer into RC did not produce significant heats. However, titration of CBM into buffer did yield significant heats, so a heat of dilution experiment of protein titrated into buffer was performed for each temperature, and the value obtained was subtracted from that from the experimental titration to correct for this effect.

The binding isotherms corrected for heat of dilution were analysed using the MicroCal ORIGIN software (version 7.0) supplied with the instrument. Based on previous depletion isotherm results with *Cc*CBM17 and *Bsp*CBM28 [7], the total concentrations of binding sites on the RC were fixed at 122.5 μM (5 mg/ml at 24.5 μmol of binding sites per g of RC) and 52.5 μM (5 mg/ml at 10.5 μmol of binding sites per g of RC) for *Cc*CBM17 and *Bsp*CBM28 respectively during the fitting process. The software provided with the instruments includes very commonly used binding models treating the acceptor (i.e. cellulose in this case) as having one class of independent binding sites or two classes of independent binding sites. In the first case, the stoichiometry (n value) represented the fraction of the total expected binding sites that acted as acceptors. In the latter case, two n values are regressed, one for each class of binding site, which represent the fractions of the total expected binding sites that are of one class or the other. C-values [$C = K_a \times A \times n$, where K_a is the association constant, A is the acceptor concentration (i.e. binding sites on the cellulose) and n is the number of binding sites on the acceptor] for the high-affinity sites were always greater than 6, allowing accurate deconvolution of the binding parameters (K_a, ΔH and n). The n value for the high-affinity site was determined during the regression and represented the fraction of the total binding sites that were high-affinity sites. $[N]_o$, an expression of the stoichiometry representing the concentration of binding sites on the cellulose, was determined by multiplying n by the total initial concentration of binding sites (i.e. 122.5 μM or 52.5 μM) and dividing by the cellulose concentration (i.e. 5 mg/ml) to give a value in μmol/g of RC. C-values for the low-affinity binding sites were to low to allow accurate deconvolution of both ΔH and n. Thus the n values were fixed at values such that the concentrations of low-affinity binding sites were equal to the values determined previously by depletion isotherms [7]. Thus ΔH and K_a could be determined for the low-affinity sites.

RESULTS AND DISCUSSION

*Cc*CBM17 and *Bsp*CBM28 recognize two classes of binding sites in RC

The cellulose depletion isotherm is a method adapted to direct measurement of the partitioning of CBM between the bound and free states when using cellulose as an insoluble sorbent. We previously studied the binding of *Cc*CBM17 and *Bsp*CBM28 to RC by this method. Binding models accounting for multiple independent binding sites on the cellulose and one-dimensional overlapping lattices of binding sites, with and without co-operativity, were discriminated using Scatchard plots and statistical evaluation of non-linear fits to the models. Based on this, it appeared that these two CBMs each recognized two independent classes of binding sites on non-crystalline cellulose [7]. Both CBMs bound completely reversibly, and there did not appear to be any co-operativity to the binding.

Titrations of the CBMs into RC yielded negative power deflections, indicating exothermic binding, and integrated isotherms of sigmoidal shape (Figure 1). These calorimetric isotherms also appeared to show two classes of binding site on RC for both CBMs. Visual inspection of the residuals from fits of a one-site binding model to the data (differences between the fit and
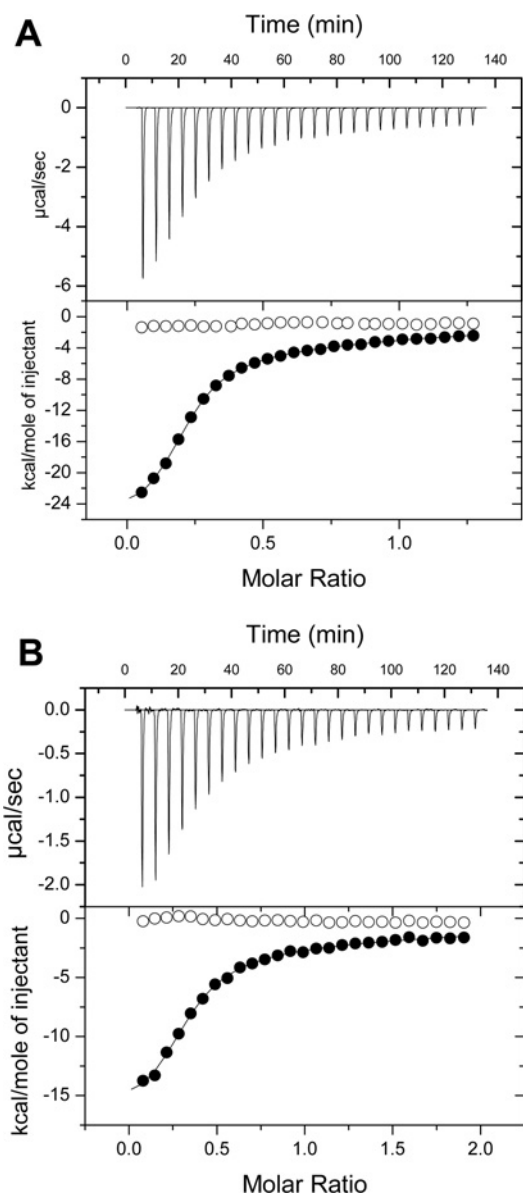
Figure 1   Representative calorimetric isotherms of binding of *Cc*CBM17 (A) and *Bsp*CBM28 (B) to RC at 25 °C and 15 °C respectively in 50 mM potassium phosphate, pH 7.0

The upper panels show the raw data, and the lower panels the integrated data. The *Cc*CBM17 and *Bsp*CBM28 titrations into RC are shown as solid circles. The lines show the fits to a two independent binding site model. The corresponding heat of dilution data (CBM titrated into buffer) are shown for reference as open circles. 1 kcal = 4.184 kJ.
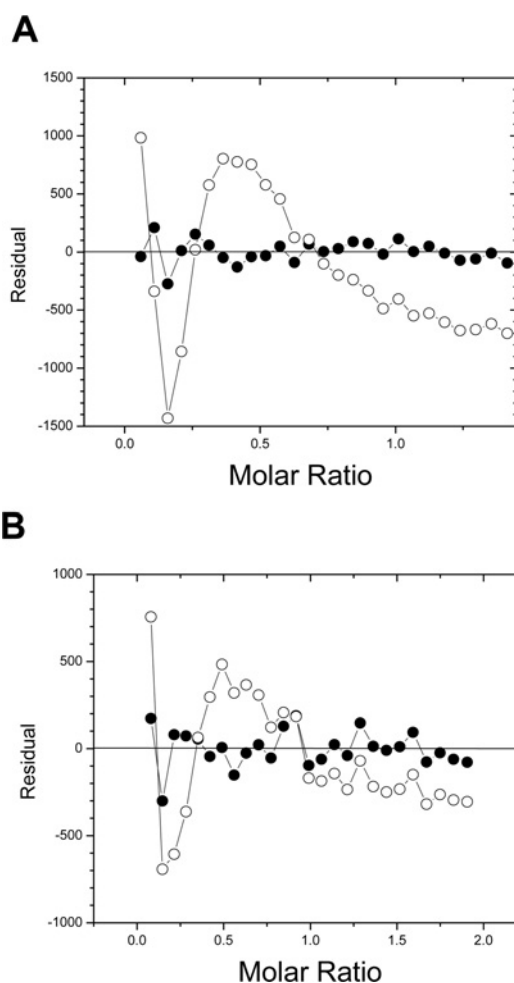

Figure 2   Residual plots of the fits of a one-site binding model (○) and a two independent binding site model (●) to the calorimetric isotherms of binding of *Cc*CBM17 (A) and *Bsp*CBM28 (B) to RC at 25 °C and 15 °C respectively

As was acknowledged previously, there may indeed be more than two classes of binding sites on RC. However, Scatchard analysis of our previous depletion isotherms showed two distinct binding phases [7]. Furthermore, fitting both the cumulative ITC isotherms (results not shown) and depletion isotherms [7] with binding models accounting for more than two independent binding sites failed to reduce the sum-of-squares of the fits and, therefore, were not statistically better. Thus, although there may be more than two distinct classes of binding sites for each of *Cc*CBM17 and *Bsp*CBM28 in RC, it is concluded that there are two dominant classes of binding sites, the general properties of which are revealed in this analysis. On this basis, all of the ITC isotherms were analysed using a two independent binding site model.

Interaction of *Cc*CBM17 with RC

The association constants for binding of *Cc*CBM17 to high- and low-affinity sites on RC were approx. 6×10^5 M^{-1} and 1×10^4 M^{-1} respectively, and agreed reasonably well with values determined previously by depletion isotherms (Table 1). The high-affinity binding site density ($[N]_o$ value) was also in acceptable agreement with that determined previously (Table 1).

At 25 °C, *Cc*CBM17 bound to cellohexaose with an affinity of 5.8×10^4 M^{-1} [$\Delta G = -27.2$ kJ/mol (-6.5 kcal/mol)], a ΔH

experimental Y values for each X value) clearly indicated systematic deviations in the one-site fit, whereas a model involving two independent binding sites gave apparently random scatter about the X-axis (Figure 2). Runs-test analysis of the residuals from the fits of a one-site binding model to the isotherms gave P-values of 0.0004 for both CBMs. Using a standard P-value threshold of 0.05, this confirmed a strong systematic deviation from the one-site model and, thus, its rejection. In contrast, a similar analysis with a two independent binding site model gave P-values of 0.212 and 0.115 (i.e. significantly greater than 0.05) for *Cc*CBM17 and *Bsp*CBM28 respectively, indicating that the two-site model was statistically appropriate.

Table 1   **Thermodynamic parameters of the interactions of *Cc*CBM17 and *Bsp*CBM28 with RC in 50 mM potassium phosphate, pH 7.0**

Values are means $\pm$ S.D. of triplicate experiments. The stoichiometry of the interaction (n) was determined as described in the Materials and methods section. $[N]_0$ indicates the concentration of binding sites per g of cellulose, and was determined as described in the Materials and methods section. N/A, not applicable. 1 cal = 4.184 J.

| CBM/method	Temp (°C)	No. of sites	$10^{-4} \times K_a$ (M^{-1})	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔS (cal/mol per K)	n	$[N]_0$ (μmol/g)
*Cc*CBM17								
ITC	15	1	60.7 + 14.0	−7.6 + 0.1	−21.3 + 0.6	−47.5 + 2.6	0.25 + 0.01	6.2 + 0.2
		2	1.2 + 0.2	−5.3 + 0.1	−8.8 + 0.3	−11.8 + 1.3	0.68	15.9
ITC	25	1	54.0 + 18.0	−7.8 + 0.2	−24.2 + 0.7	−55.0 + 3.1	0.23 + 0.00	5.5 + 0.0
		2	1.2 + 0.4	−5.5 + 0.2	−10.3 + 1.1	−15.9 + 4.5	0.68	15.9
ITC	35	1	59.6 + 0.4	−8.1 + 0.0	−21.5 + 0.5	−43.4 + 1.7	0.25 + 0.01	6.2 + 0.2
		2	1.3 + 0.3	−5.8 + 0.2	−10.5 + 0.9	−15.5 + 3.5	0.68	15.9
Depletion*	25	1	113.0 + 0.1	−8.4 + 0.3	N/A	N/A	N/A	8.6 + 1.4
		2	1.8 + 0.5	−5.9 + 0.4	N/A	N/A	N/A	15.9 + 1.3
ITC – G6†	25	–	5.9 + 0.1	−6.6 + 0.0	−14.5 + 0.0	−27.5 + 0.7	1	N/A
*Bsp*CBM28								
ITC	15	1	44.6 + 2.4	−7.3 + 0.3	−19.3 + 2.7	−41.7 + 10.4	0.26 + 0.02	2.7 + 0.3
		2	2.0 + 0.3	−5.7 + 0.1	−7.3 + 2.3	−5.6 + 7.6	0.65	6.8
Depletion*	25	1	99.0 + 0.2	−8.3 + 0.4	N/A	N/A	N/A	3.7 + 0.4
		2	2.1 + 0.1	−5.9 + 0.4	N/A	N/A	N/A	6.8 + 0.6
ITC – G6‡	25	–	4.0 + 0.1	−6.4 + 0.0	−14.9 + 0.0	−28.6 + 0.5	1	N/A

* Taken from [7] and shown for reference.
† Parameters of binding of *Cc*CBM17 to cellohexaose (G6) were determined by ITC and taken from [5] for reference.
‡ Parameters of binding of *Bsp*CBM28 to cellohexaose (G6) were determined by ITC and taken from [6] for reference.

of −60.7 kJ/mol (−14.5 kcal/mol) and a ΔS of −113 J/mol per K (−27 cal/mol per K) [5] (Table 1). Thus at this temperature the recognition of the high-affinity site in RC by *Cc*CBM17 is less entropically favourable by approx. −117 J/mol per K (−28 cal/mol per K) [or $T\Delta\Delta S$ of approx. −35.1 kJ/mol (−8.4 kcal/mol)] than the recognition of cellohexaose (Table 1). The improvement in ΔG [approx. −5.4 kJ/mol (−1.3 kcal/mol)] comes by virtue of an interaction that is enthalpically more favourable by approx. −40.6 kJ/mol (−9.7 kcal/mol). Thus, if the loss of configurational entropy upon binding RC is less than for cellohexaose, this effect is eclipsed by other less favourable contributions to entropy and more favourable contributions to enthalpy.

The dependence of ΔH on experimental temperature for the recognition of the high-affinity sites on RC by *Cc*CBM17 was not linear (Figure 3). There was a decrease in ΔH when going from 15 °C to 25 °C, giving a ΔC_p (change in heat capacity) of −1213 ± 377 J/mol per K (−290 ± 90 cal/mol per K). The increase in ΔH when going from 25 °C to 35 °C gave a ΔC_p of 1130 ± 377 J/mol per K (270 ± 90 cal/mol per K). Such non-linear behaviour of ΔC_p has been documented for an antibody–carbohydrate interaction [11] and was suggested to arise from changes in the antibody conformation [12]. The conformation of *Cc*CBM17 is identical in its liganded and unliganded states [5]; furthermore, *C*cCBM17 showed a linear relationship between ΔH and temperature for binding to cello-oligosaccharides [5]. Therefore it is unlikely to be a result of structural changes in the CBMs, but it is possible that the behaviour of ΔC_p, in this case, represents a structural change in the RC.

The concept of structural rearrangements in cellulose upon CBM binding is not a new one: *Cf*CBM2a was observed to release cellulose fragments non-catalytically from crystalline cellulose [13]. The mechanism by which this occurs is unknown, but it is assumed to be via mechanical disruption of the cellulose fibres [14]. In the case of *Cc*CBM17 binding, a structural rearrangement in the cellulose may occur as changes in inter- or intra-molecular hydrogen bonding while the cellulose chain(s) are induced to adopt a different conformation. Such a possibility implies that the recognition of the high-affinity sites may occur via a multi-step
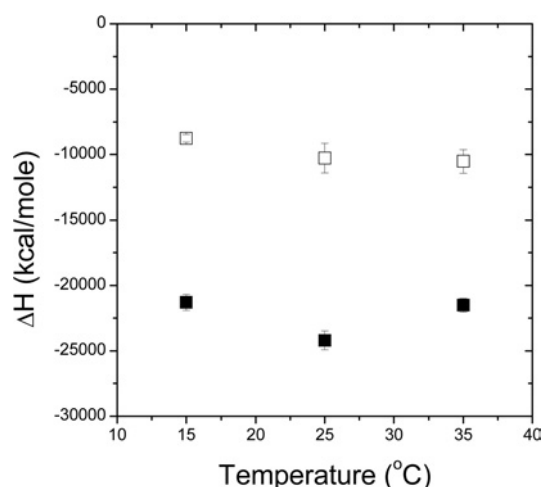
Figure 3   Enthalpy against temperature data for binding of *Cc*CBM17 to RC in 50 mM potassium phosphate, pH 7.0

■, High-affinity binding sites; □, low-affinity sites. Error bars represent the S.D. of triplicate experiments. 1 kcal = 4.184 kJ.

equilibrium. It is not possible to determine conclusively if this is in fact true and, in this event, the parameters reported for this binding site would omit details of the individual steps required to form the bound species. However, the values reported would, to a first approximation, give a general representation of energetics involved in the overall equilibrium and, thus, the interpretation would remain the same.

The recognition of the low-affinity site in RC by *Cc*CBM17 is more entropically favourable by ∼46.0 J/mol per K (∼11 cal/mol per K) [or T$\Delta\Delta S$ of ∼13.8 kJ/mol (∼3.3 kcal/mol)] and ∼17.6 kJ/mol (∼4.2 kcal/mol) less enthalpically favourable than the recognition of cellohexaose, to give a differential in ΔG of approx. 4.2 kJ/mol (1 kcal/mol) (Table 1). ΔH for this site appeared to vary approximately linearly ($R^2 = 0.87$) with

temperature (Figure 3), yielding a $\Delta C_{p,RC}$ of -368 ± 146 J/mol per K (-88 ± 35 cal/mol per K), compared with a $\Delta C_{p,G6}$ of -611 ± 46 J/mol per K (-146 ± 11 cal/mol per K) for the recognition of cellohexaose. The magnitude of ΔC_p is thought to be the best indicator of ΔS_{solv}, the contribution of solvent rearrangement to entropy, where a large negative ΔC_p generally indicates a more favourable ΔS_{solv} [15]. Based on this generalization, because $\Delta C_{p,RC}$ is greater than $\Delta C_{p,G6}$, then ΔS_{solv} for RC must be less than ΔS_{solv} for cellohexaose, meaning the contribution of ΔS_{solv} to binding at the low-affinity RC site is less favourable than for cellohexaose binding. Overall, ΔS can be generally be considered the sum of ΔS_{solv} and $\Delta S_{config/rot/trans}$, where $\Delta S_{config/rot/trans}$ is change in entropy resulting from alterations in the configurational, rotational and translational freedom of the system components [15]. Because the difference between the ΔS_{solv} values for RC and cellohexaose is negative, but the overall difference between ΔS values is positive [$+46$ J/mol per K ($+11$ cal/mol per K)], the difference between $\Delta S_{config/rot/trans}$ values for binding to RC compared with cellohexaose must be substantially positive to overcome the deficit of ΔS_{solv}. Thus the change in entropy due to the changes in the configurational, rotational and translational freedom of the system components is more favourable for binding RC than for the recognition of cellulose. Although this analysis is somewhat rough due to the limited precision of the experimental measurements, this does suggest consistency with the assumption of less loss of configurational entropy when binding to the low-affinity site in insoluble cellulose. However, this appears to be at the cost of an overcompensating change in ΔH which results in a poorer ΔG of binding.

Interaction of *Bsp*CBM28 with RC

Due to the low density of binding sites for *Bsp*CBM28 on RC, the inability to use RC in the calorimeter at concentrations greater than those used, and the resulting difficulty in maintaining adequate *C*-values, ITC using this CBM was less tractable. However, the appropriate conditions were met when performing the experiments at a sufficiently low temperature, i.e. 15 °C. As with *Cc*CBM17, the calorimetric values of K_a and $[N]_o$ for binding of *Bsp*CBM28 to the high-affinity site were similar to those determined by depletion isotherms at 25 °C (Table 1).

At 25 °C, *Bsp*CBM28 bound to cellohexaose with an affinity of 4.0×10^4 M^{-1} [$\Delta G = -26.4$ kJ/mol (-6.3 kcal/mol)], a ΔH of -61.1 kJ/mol (-14.6 kcal/mol) and a ΔS of -117 J/mol per K (-28 cal/mol per K) [6]. Although the parameters for binding of *Bsp*CBM28 to RC were determined at 15 °C, it is clear that the ΔH for the high-affinity site on RC was substantially more favourable and the ΔS substantially less favourable in comparison with binding to cellohexaose (Table 1), a pattern identical with that observed for *Cc*CBM17. Likewise, the pattern for binding at the low-affinity site was the same, i.e. a less favourable ΔH and more favourable ΔS in comparison with cellohexaose. The thermodynamic signatures of cello-oligosaccharide binding by *Cc*CBM17 and *Bsp*CBM28 are extremely similar; it would also appear that the thermodynamic signatures for RC recognition by the two CBMs are very similar.

Conclusions

*Cc*CBM17 and *Bsp*CBM28 are CBMs that each recognize two distinct classes of binding sites on RC: one of high affinity and one of low affinity. Both classes are recognized via enthalpically driven processes at the temperatures tested. Contrary to assumption, the high-affinity interactions with RC are 10–100-fold stronger relative to those for cello-oligosaccharide binding due to gains in ΔH, not gains in configurational ΔS. The individual contributors to the thermodynamic parameters remain unknown, as structural rearrangements in the cellulose are a possibility, leaving us with the question of how the gain in ΔH is generated. Is this a result of differences in more favourable direct CBM–cellulose interactions, such as hydrogen bonding? Or does it somehow result indirectly from a thermodynamically favourable structural rearrangement in the cellulose? We currently have insufficient evidence to comment. In contrast with binding at the high-affinity site, the low-affinity interaction does appear to have gains in configurational entropy, but with a compensating enthalpic loss.

Although this study does suggest a thermodynamic basis for the tight binding of CBMs to RC, it also highlights that the structure of non-crystalline cellulose is largely unknown, making it difficult to associate these energetic observations with structural features. Indeed, even the aetiology of the multiple classes of binding sites on RC remains unclear. The presence of particular 'micro-structures' in non-crystalline cellulose and their solvation may have effects on binding of which we are currently poorly informed. In short, although we have made strides in the last 10 years, there is much to learn about how CBMs interact with both non-crystalline and crystalline cellulose.

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