The cellulose-binding domain of the major cellulobiohydrolase of *Trichoderma reesei* exhibits true reversibility and a high exchange rate on crystalline cellulose

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Communicated by T. Kent Kirk, U.S. Department of Agriculture Forest Products Laboratory, Madison, WI, August 5, 1996 (received for review June 10, 1996)

**ABSTRACT** Cellulose-binding domains (CBDs) bind specifically to cellulose, and form distinct domains of most cellulose degrading enzymes. The CBD-mediated binding of the enzyme has a fundamental role in the hydrolysis of the solid cellulose substrate. In this work we have investigated the reversibility and kinetics of the binding of the CBD from *Trichoderma reesei* cellulobiohydrolase I on microcrystalline cellulose. The CBD was produced in *Escherichia coli*, purified, and radioactively labeled by reductive alkylation with H. Sensitive detection of the labeled CBD allowed more detailed analysis of its behavior than has been possible before, and important novel features were resolved. Binding of the CBD was found to be temperature sensitive, with an increased affinity at lower temperatures. The interaction of the CBD with cellulose was shown to be fully reversible and the CBD could be eluted from cellulose by simple dilution. The rate of exchange measured for the CBD-cellulose interaction compares well with the hydrolysis rate of cellulobiohydrolase I, which is consistent with its proposed mode of action as a processive exoglucanase.

Cellulose is a polymer composed of α-glucose units linked by β-1,4-glycosidic bonds. The cellulose chains can pack together to form fibers, which are crystalline in nature (1). This solid and ordered substrate poses some special requirements on the enzymes hydrolyzing it. Most cellulose degrading enzymes have a bifunctional organization composed of two domains connected by a linker region. One of the domains is dedicated for binding to the solid cellulose and is called a cellulose-binding domain (CBD). The other domain contains the catalytic machinery. These catalytic domains have been grouped into several families sharing the same basic structure and the same chemical reaction mechanism (2). *Trichoderma reesei* cellulobiohydrolase I (CBHI) is an exoglucanase with a long tunnel-shaped active site (3). CBHI and other exoglucanases initiate the hydrolysis at the chain ends, and do not produce significant amounts of new chain ends on the cellulose surface (4). They seem to act by a processive mechanism hydrolyzing consecutive bonds along the same chain without dissociating (2, 3). The endoglucanases cleave internal bonds along the cellulose chains probably by cycles of adsorption and desorption.

Based on sequence comparisons the CBDs have been grouped into several families (5). To date, the three-dimensional structures of two CBDs have been published. One is from *T. reesei* CBHI (6) and the other from *Cellulomonas fimi* xylanase/cellulobiohydrolase Cex (7). Both the fold and the size of these CBDs are different, but a common pattern of aromatic residues and amides is found at the surface of both proteins. Many experiments have shown that this surface is the primary interaction site between a CBD and cellulose (7–10). Many studies directly correlate the efficiency of crystalline cellulose degradation to the binding efficiency of the enzyme (11) and the removal of a CBD typically results in a decrease of about 50–80% of the activity of a given cellulase on solid but not on soluble substrates (12, 13).

Studies carried out mainly with fungal cellulases indicate that once adsorbed, harsh conditions are required for their desorption (14–16). This has led to the suggestion that the binding is irreversible (17–19). An exchange between bound and free cellulase has been demonstrated, but no desorption of enzyme was noted as a result of dilution (19). Because catalytic domains can be eluted from cellulose and much of the affinity of the complete enzyme is mediated by its CBD (8, 17, 20), it has been proposed that the CBD is responsible for the apparently irreversible binding (17, 21). Because this binding will also dictate the mobility of intact cellulases at the cellulose surface, the question of the binding equilibrium is of key importance for understanding their action. Moreover, many potential applications of cellulases depend on enzyme recycling or the use of CBDS to immobilize recombinant proteins, and require that the adsorption of CBDS is understood and can be controlled. Using a H-labeled CBD from *T. reesei* CBHI, we have now been able to show for the first time that its binding is fully reversible with elution occurring by simple dilution. The dynamics of the binding was also investigated and implications of these results on the function of CBHI are discussed.

**MATERIALS AND METHODS**

**Production and Purification of CBD.** The CBHI CBD was produced in *Escherichia coli* as a double CBD containing the N-terminal CBD of *T. reesei* cellulobiohydrolase II (CBHII) joined by a linker to the C-terminal CBD of CBHII as described (22). Standard protocols were used for all DNA manipulations (23). In brief, the expression plasmid was based on the vector pKK223-3 with the lac promoter and the pelB signal sequence of *Erwinia carotovora* (24). The expression cassette contained the cDNA segments coding for the 38 amino acids of the CBHII CBD, 3 amino acids of the CBHII linker (plasmid pTTc9) (25), 21 residues of the CBHI linker, and finally the 36 residues of the CBHI CBD (plasmid pTTc1) (26). Production of the resulting double CBD was carried out in a 1.5 liter fermentor, and constant pH and dissolved oxygen levels were maintained throughout the fermentation. Isopropyl-β-D-thiogalactopyranoside was added (0.5 mM, 15–20 h after inoculation) to induce expression and the cultivation was continued until the double CBD secreted to the periplasmatic space leaked to the culture medium. It was purified by

*Abbreviations:* CBD, cellulose-binding domain; CBHI, cellulobiohydrolase I; BMCC, bacterial microcrystalline cellulose.

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hydrophobic interaction chromatography (Butyl Sepharose 4B, Pharmacia) and preparative reversed-phase chromatography using Source RPC media (Pharmacia) with a water/acetonitrile gradient. Both liquid phases contained 0.1% tri-fluoroacetic acid. The CBHI CBD was cleaved from the double CBD fusion protein by trypsin, purified by reversed-phase chromatography and lyophilized.

**Labeling of CBD with ³H.** The ³H labeling was performed by reductive methylation essentially as described by Jentoft and Dearborn (27). Lyophilized peptide (6.6 mg) was dissolved in 2.4 ml of 100 mM Hepes buffer (pH 7.5). Three hundred microliters of 10 mM formaldehyde and 10 mCi (1 Ci = 37 GBq) of ³H-enriched NaCNBH₄ (0.86 µmol) (TRK708, Amersham) were added and the mixture was incubated for 3 h at 22°C. The reaction was terminated and unreacted reagents were separated by repurifying the peptide by reversed-phase chromatography as described above.

**Characterization of the CBD.** The proteolytically cleaved and purified CBHI CBD peptide, as well as the methylated CBD, were analyzed by matrix-assisted laser desorption ionization/time-of-flight (MALDI/TOF) mass spectroscopy. To assess the purity of the methylated CBD it was also analyzed by reversed-phase chromatography as described above.

**Determination of Binding Isotherms.** Stock solutions of CBD were prepared at approximately 20 µM by dissolving lyophilized peptide with 50 mM acetate buffer (pH 5.0) containing 50 mM NaCl. The concentration of the solutions was determined by UV adsorption at 280 nm using the molar extinction coefficient 5545 cm⁻¹ M⁻¹ (9). Dilutions of the stock solutions were then made with the same acetate buffer, but containing 1% bovine serum albumin (BSA). One hundred microliters of the peptide dilution was then mixed with an equal volume of a suspension (2 g/liter) of bacterial microcrystalline cellulose (BMCC) in water. The suspension was mixed in a test tube with a small magnetic bar for 3 h. The samples were then filtered through Millex GV 13 (0.22 µm) filter (Millipore) cartridges and the amount of ³H was quantified with a liquid scintillation counter. Due to the high off-rate of the CBD, all steps of the binding experiments, including filtration, were performed in a carefully thermostated environment. Quantification of the nonmethylated CBD was performed using reversed-phase high performance liquid chromatography (HPLC) (9, 10) and with no BSA added. Concentrations were calculated using standard curves. The BMCC was grown and prepared as described (28).

**Measurement of Nonequilibrium Adsorption and Desorption.** The time required to achieve equilibrium was measured by mixing ³H-labeled CBD and cellulose with a magnetic stirrer (volumes as above) and then terminating the adsorption by fast filtration. Nonequilibrium desorption from cellulose was measured by first allowing a series of identical cellulose–CBD mixtures to achieve equilibrium. The equilibrium concentration was determined by analyzing two of the samples. Then a 2–10 time volume of acetate buffer (0.5% BSA) without CBD was added. Samples were filtered and analyzed at different times to determine how fast the new equilibrium was established.

**Exchange Rate of the CBD at the Cellulose Surface at Equilibrium.** To determine the exchange rates at equilibrium, bound ³H-labeled CBD was allowed to compete for binding with nonlabeled CBD and vice versa. One hundred microliters of BMCC suspension and 100 µl of ³H-labeled CBD solution (2 µM) were allowed to reach equilibrium. Then the same molar amount of nonlabeled CBD was added. The concentration of the added CBD equaled the concentration of the free ³H-labeled CBD. In this way the equilibrium as a whole was not affected, and by measuring how fast the amount of bound ³H-labeled CBD was halved we could calculate the exchange rate of the CBD on the cellulose surface. The experiments were also performed so that nonlabeled CBD was first adsorbed and ³H-labeled CBD was then added. Experiments at different temperatures were performed using carefully thermostated conditions.

**RESULTS**

**Purification and Labeling of the CBD Peptide.** The CBHI CBD used in this work was produced by proteolytic cleavage of a double CBD, which contains the CBHI and CBBD CDs joined by parts of their natural linker peptides. Fig. 1 shows the amino acid sequence of the isolated CBHI CBD with the remaining 11 amino acid residues of the linker. This linker segment is not regarded as a part of the CBD and does not affect its binding to cellulose (6, 29). Reversed-phase chromatography and MALDI/TOF mass spectroscopy confirmed both the purity and identity of the isolated protein, the calculated mass being 4696.63 Da and the experimentally measured one 4695.34 Da. The methyl groups introduced in the tritium labeling are added to amines. Only one amine, which is at the N terminus, is available in the peptide and, because the N-terminal residue is a proline, only one methyl group is added to each CBD. This was confirmed by mass spectroscopy, which also showed that all of the peptide in the reaction mixture had reacted, since no nonmethylated CBD was detectable in the mass spectrum of the product. Scintillation counting of the fractions collected from a reversed-phase chromatography run revealed about 1% of the radioactivity in the flow-through and the rest was associated with the single peak containing the CBD. The specific activity of the methylated CBD was 1.38 Ci/mmol. The N terminus is located 10 amino acid residues away from the folded part of the peptide, which interacts with cellulose, which minimizes the risk of the methylation interfering with the binding. This was confirmed by a comparison of the binding isotherms of methylated and nonmethylated CBD, which revealed no significant differences in their binding behavior (Fig. 2).

**Optimization of the Assay Conditions.** The improved sensitivity and accuracy gained by the ³H-labeling permitted a much more detailed analysis of the binding behavior of the CBHI CBD than has been possible before. If HPLC is used for detection concentrations down to 0.3–1 µM can be measured, but other proteins cannot be added to prevent nonspecific adsorption of the CBD (9, 10). Using ³H-labeled CBDs and scintillation counting for quantification the assay is not disturbed by the presence of, e.g., BSA to prevent nonspecific adsorption. In our hands, addition of 0.5% of BSA allowed CBD concentrations in the nanomolar range to be measured easily. BSA did not influence the CBD binding to cellulose at concentrations greater than 1–2 µM, but below this specific binding of the CBD to container walls etc., increased clearly in the samples without BSA. Another advantage of using BSA is that the adsorption experiments can be terminated by rapid filtration instead of centrifugation. Less than 1% of the protein was nonspecifically bound to the filter membrane during filtration, and more accurate time points were obtained to follow the binding kinetics.

**Binding Properties of the CBHI CBD.** The temperature dependence of the equilibrium formed is evident from the

**Fig. 1.** Sequence of the CBHI CBD used in this study. The first 11 amino acids are a part of the linker that forms an extended chain between the CBD and catalytic domain in the native enzyme. The last 36 amino acids fold into the rigid disulfide-stabilized CBD.
binding isotherms shown in Fig. 3. At lower temperatures the equilibrium is clearly shifted toward the bound state. The differences in affinity can be quantified by measuring the initial slopes, $K_p$, of the isotherms (Table 1).

The nonequilibrium desorption experiment presented in Fig. 4 is a critical test for the reversibility of adsorption. The resulting data shows that the "ascending" isotherm is the same as the "descending" isotherm (30), which is a requirement for a reversible adsorption. The same result was obtained irrespective of dilution ratio. The reversible binding of the CBD was also apparent in our initial attempts to quantify bound CBD by measuring the radioactivity of the cellulose. Elution of the CBD occurred during the washing steps of the membrane used to collect the cellulose and the amount of bound CBD could not be measured directly from the cellulose.

In agreement with the evidence for reversibility, we were also able to measure an exchange rate for the CBDs on the cellulose surface at equilibrium. Fig. 5 shows the data obtained at different temperatures. The curves for binding and desorption of $^3$H-labeled CBD should be mirror images since both are measured at equilibrium. Single exponential curves were fitted to the data giving the rate of adsorption and desorption at equilibrium. The first order model was used for all data since the quality of the data does not warrant the use of more complicated models. The rate constants are shown in Table 1. The rate shows a clear temperature dependency with approximately a 2-fold increase for every 10°C rise in temperature.

**DISCUSSION**

Cellulase adsorption and desorption is a central issue in attempts to explain cellulase function. Fungal and bacterial cellulose-binding domains have also received much attention due to their many interesting potential applications (5). Our current data provide new insight into the roles and function of a fungal CBD and explain some of its properties that have been in dispute. The following discussion concerns the family 1 CBDs (5), however, and the conclusions drawn are not necessarily valid for CBDs belonging to other families.

Temperature dependence of the binding of cellulases has been addressed in several earlier studies and both increased (31) and decreased (9) binding with decreasing temperature has been reported. Our data show very clearly that the isolated CBHI CBD binds with a higher affinity at lower temperatures. The enthalpy of adsorption can be derived from the temperature dependency of $K_p$ using the van’t Hoff equation, which assumes constant heat capacity (32). Although we cannot prove constant heat capacity, the temperature dependence observed here is consistent with binding dominated by the enthalpy factor. This also agrees with data from other similar interactions (33–35).

The key finding of our current work, presented in Figs. 4 and 5, is the demonstration that the binding of the CBD is a readily reversible event. It immediately follows that the CBD alone is not responsible for the apparently irreversible binding of intact cellulases as suggested earlier (17, 21). The explanation may be found in the two-domain organization of the enzyme. Covalent linkage of the two domains results in binding interactions that are mutually interdependent. Binding of one domain increases the effective concentration of the other domain. This makes the interaction of the second domain with the surface much more probable (36), which in turn can increase its affinity (22). Especially in CBHI, productive binding of the cellulose chain into the long active site tunnel of the catalytic domain could also contribute to the unusually tight binding of the intact enzyme. Another factor that very probably contributes to the observed irreversible binding of cellulases is nonspecific adsorption. Here BSA was used to prevent nonspecific adsorption and at low CBD concentrations below 1 μM nonspecific adsorption was a serious problem if BSA was not added. At higher concentrations nonspecific adsorption was not significant on the pure bacterial cellulose. However, in natural

**Table 1. Values for temperature dependency of the exchange rate ($k$) and partition coefficient ($K_p$)**

<table>
<thead>
<tr>
<th>$T$, °C</th>
<th>$K_p$, 1g⁻¹</th>
<th>$k$, s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>4.9 ± 0.4</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>22</td>
<td>2.1 ± 0.2</td>
<td>0.012 ± 0.0025</td>
</tr>
<tr>
<td>30</td>
<td>1.3 ± 0.1</td>
<td>0.029 ± 0.003</td>
</tr>
</tbody>
</table>

![Fig. 2. Isotherm showing that methylated (●) and nonmethylated (■) CBD bind identically to BMCC. The experiment was performed at 22°C.](image)

![Fig. 3. Binding isotherms at 4°C (●), 22°C (■), and 30°C (○). The figure shows a clear temperature dependency on the equilibrium positions for the binding of CBHI CBD to BMCC.](image)

![Fig. 4. Reversibility at nonequilibrium. Part of the equilibrium isotherm at 4°C (●) is shown. A time course measurement (□) shows how the CBD returns to equilibrium when it has been disturbed by dilution with buffer.](image)
substracts the presence of other components such as lignin and hemicellulose may increase nonspecific adsorption of cellulases.

The tight binding of the exoglucanases has been particularly difficult to explain in terms of their mechanistic function on crystalline cellulose. If an exoglucanase indeed bound practically irreversibly, how could it work processively and move along the cellulose chains as the hydrolysis proceeds? Does it need to “hydrolyze itself off” the cellulose to desorb? Two parameters described in this paper contribute to our understanding of the function of the intact enzyme. These are the exchange rate and the partitioning of the CBD between the cellulose surface and liquid phase, which describe how large a fraction of the CBDs is bound to the cellulose and what is their mobility at the surface. It is assumed that the initial contact between the cellulose and an exoglucanase is through the CBD. With a fast exchange rate and a low partitioning the enzyme could then leave the cellulose before the catalytic domain has had time to find a free cellulose chain end to hydrolyze. On the other hand, with a slow exchange rate the enzyme would be adsorbed to the surface for a long time. If no chain ends are available this would result in nonproductive binding.

Fig. 5. Temperature dependence of the exchange-rate of the CBD on the cellulose surface. Experiments were performed so that equilibrium was allowed to be established with 3H-labeled CBD. Then an equal molar amount of nonlabeled CBD was added, at the same concentration as the free 3H-labeled CBD. In this way the equilibrium as a whole was not disturbed. The amount of bound 3H-labeled CBD was then determined as a function of time (x). The experiment was repeated in the reverse order (+).

We thank Dr. Mike Penner and Dr. Olle Teleier for valuable comments on the manuscript. This work was supported by grants from the Academy of Finland and the Technology Development Center of Finland.