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Effects of pH and High Ionic Strength on the Adsorption and Activity of Native and Mutated Cellobiohydrolase I From *Trichoderma reesei*

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ABSTRACT **Cellobiohydrolase I (CBHI) is the major cellulase of *Trichoderma reesei*. The enzyme contains a discrete cellulose-binding domain (CBD), which increases its binding and activity on crystalline cellulose. We studied cellulase–cellulose interactions using site-directed mutagenesis on the basis of the three-dimensional structure of the CBD of CBHI. Three mutant proteins which have earlier been produced in *Saccharomyces cerevisiae* were expressed in the native host organism. The data presented here support the hypothesis that a conserved tyrosine (Y492) located on the flat and more hydrophilic surface of the CBD is essential for the functionality. The data also suggest that the more hydrophobic surface is not directly involved in the CBD function. The pH dependence of the adsorption revealed that electrostatic repulsion between the bound proteins may also control the adsorption. The binding of CBHI to cellulose was significantly affected by high ionic strength suggesting that the interaction with cellulose includes a hydrophobic effect. High ionic strength increased the activity of the isolated core and of mutant proteins on crystalline cellulose, indicating that once productively bound, the enzymes are capable of solubilizing cellulose even with a mutagenized or with no CBD.**

Key words: cellulase, cellulose-binding domain, mutagenesis, substrate binding, cellulose degradation, *Trichoderma reesei*

INTRODUCTION

Practically all cellulases and other glycosyl hydrolases degrading insoluble polysaccharides possess a separate substrate-binding domain which facilitates breakdown of the substrate.[1,2] On the basis of amino acid sequence comparisons of cellulolytic enzymes, five families of cellulose-binding domains (CBD) have been identified.[3] The molecular size within a given CBD family is maintained, but differs considerably between families. Typically, the CBDs of bacterial cellulases and xylanases contain 100–150 and the fungal CBDs 30–40 amino acid residues. There is no apparent sequence similarity between these two CBD types but the conservation of certain aromatic amino acids within a sequence family highlights their importance in cellulose recognition and binding. The three-dimensional structure of the CBD from the filamentous fungus *Trichoderma reesei* cellobiohydrolase I (CBHI) has been solved by NMR, revealing an amphiphilic wedge-shaped structure with two relatively flat surfaces.[4] This general structure is thought to be shared by all fungal CBDs due their high sequence similarity.[5]

N- or C-terminal CBDs or CBDs between two catalytic domains are usually separated from the catalytic domains by linker peptides rich in proline, threonine, and serine. The linker peptide is often *O*-glycosylated, which probably helps to maintain its extended conformation and may also protect the extended linker from proteolysis. It has been shown that separation between the cellulase domains is important for full catalytic activity, although the exact distance is not critical for productive interaction and hydrolysis of cellulose.[6,7] It has been suggested that a separate binding domain allows the enzymes to perform catalytic cycles while remaining attached to the substrate. In addition, it has been postulated that the cellulose-binding domains of cellulases may have a destabilizing effect on the cellulose structure or facilitate the solubilization of individual glucose chains released from cellulose crystals during hydrolysis.[8,9]

Hydrogen bonds, van der Waals forces, and aromatic ring polarization attractions contribute to the interaction between the proteins and carbohydrates.[10–12] Structure-based site-directed mutagenesis studies have indicated that conserved aromatic amino acids are essential for the function of both

Abbreviations used: CBHI, cellobiohydrolase I; CBD, cellulose-binding domain; DNS, dinitrosalisylic acid; EtOH, ethanol; SDS, sodium dodecyl sulfate; cenA, endoglucanase A; Cex, exoglucanase.

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bacterial and fungal CBDs.[13,14] It has also been suggested that hydrophobic interactions play a role in the binding.[15] However, detailed studies describing these interactions have not hitherto been presented.

In this work we studied the interactions between cellulose and mutated CBDs of *T. reesei* CBHI. Three mutant proteins, shown earlier to have altered binding properties to cellulose,[13] were expressed in the native host organism. It was readily observed that the properties of the mutant enzymes were different from those expressed in the heterologous host. Furthermore, the effects of pH and ionic strength on CBHI binding and activity were studied in detail. The mode of interaction between the CBD and crystalline cellulose is discussed, on the basis of the effects observed in activity and adsorption parameters.

MATERIALS AND METHODS

Strains and Vectors

E. coli strain DH5α [F$^-$, *end*A1, *hsd*R17 (r_{K^-}, m_{K^+}), *sup*E44,*thi*-1, λ$^-$, *rec*A1, *gyr*A96, *rel*A1, Δ(*arg*-F*lac*ZYA)U169, ϕ80d*lac*ZΔM15] was used as the bacterial cloning host. The *T. reesei* strain VTT-D-93201, a derivative of strain QM9414 lacking the *cbh1* gene, was used as the expression host. The vector used in *Trichoderma* transformations was the plasmid pEM-F5, a derivative of pUC18 containing CBHI cDNA[16] linked to the CBHI promoter and terminator sequences (E. Margolles, unpublished). A phleomycin selection plasmid pAN8-1[17] was used in the selection of the *Trichoderma* transformants.

DNA Techniques

The cDNA of *cbh1* was mutagenized as described elsewhere.[13] A *Bst*EII/*Sma*I restriction fragment in plasmid pEM-F5 was exchanged with the fragment containing the mutated region and the resulting plasmid was transformed to *E. coli* DH5α. Standard methods were used in the DNA manipulations.[18] *Trichoderma* transformation was carried out by the method of Penttilä et al.[19] as described elsewhere.[7]

Protein Purification

The mutant proteins were produced as described by Srisodsuk et al.[7] in a medium containing 2% Solca Floc, 1% distillers spent grain, 1.5% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 5 mg/liter $FeSO_4 \cdot 7H_2O$, 1.6 mg/liter $MnSO_4$, 1.4 mg/liter $ZnSO_4 \cdot 7H_2O$, and 3.7 mg/liter $CoCl_2 \cdot 6H_2O$. The protein was purified essentially according to Reinikainen et al.[13] except that the DEAE chromatography was performed in 50 mM NaAc, pH 5.6. The purity of CBHI protein was checked by SDS-PAGE.[20] The CBHI core was produced by proteolytic cleavage with papain as described earlier.[21]

Western Blotting

The proteins were transferred electrophoretically onto nitrocellulose membrane.[22] Cellulases were detected with monoclonal antibodies raised against CBHI (CI-89, C271,[23] and H-4[24]), and thereafter recognized by commercial alkaline phosphatase labelled goat anti-mouse IgG (Sigma). Staining was carried out with the Protoblot® kit (Promega).

Enzyme Activity Measurements

McIlvaine phosphate-citrate buffers were used in all experiments. The ionic strength was measured by conductance and adjusted to be equal in all buffers by dilution to a value corresponding to that in 50 mM pH 2.2 McIlvaine buffer; 0.25 mM methylumbelliferyl cellobioside was used to determine the enzyme activity on a soluble substrate, as described previously.[25–27]

Acetobacter xylinum microcrystalline cellulose (prepared as described in 28) was used to determine the enzyme activity on crystalline cellulose. Cellulose suspension (1 g/liter) was shaken at 50°C with the enzyme solution (2.1 μM) for different periods. The formation of reducing sugar was determined by the dinitrosalisylic acid (DNS) method[29] using glucose as standard and the activity was calculated from the rate of reducing sugar formation. Alternatively the end products were analyzed by HPLC. The hydrolysis reaction was stopped at timed intervals by adding half the reaction volume of a stopping reagent consisting of 9 parts 94% EtOH and 1 part 1 M glycine pH 11. The cellulose was centrifuged down and soluble oligosaccharides in the supernatant were analyzed by HPLC using a Hamilton HC-40 column and refractive index detection (Waters 410). The mobile phase was water and deashing cartridges (Micro-Guard, BIO-RAD) were used as precolumn.

Adsorption Studies

Enzyme solution (0.025–15 μM) was mixed with 1 g/liter cellulose suspension and incubated at 4°C for 90 min by vigorous shaking in a gel shaker (Heidolph). The cellulose-bound enzyme was separated by filtration through 0.22-μm Durapore membranes (Millipore, USA). The free enzyme was quantitated by spectrophotometry at 280 nm or by spectrofluorometry (Shimadzu RF-5000, Japan) at an excitation wavelength of 280 nm (band width 3 nm) and an emission wavelength of 350 nm (band width 20 nm). A separate standard curve was prepared for each mutant enzyme. The enzyme concentration was based on the molar extinction coefficient $\varepsilon = 73000\ M^{-1}\ cm^{-1}$.[30] The amount of bound enzyme was calculated from the difference between initial enzyme and free enzyme concentrations.

The adsorption data were analyzed according to the model previously presented.[28] The dependence

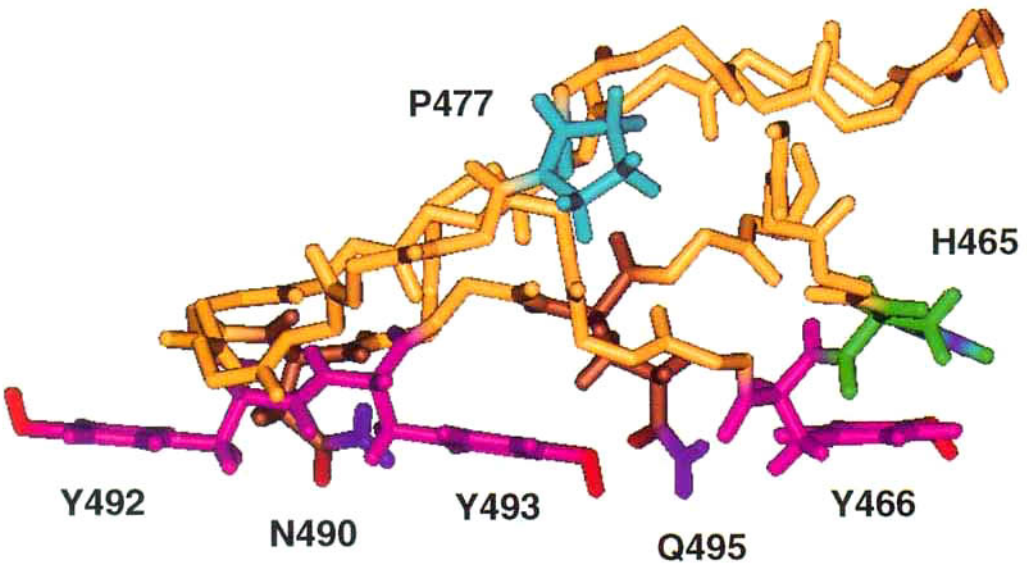

Fig. 1. The CBD of CBHI is a 36 amino acid peptide which folds into a wedge-shaped structure with overall dimensions of 31 × 18 × 10 Å. One hydrophilic face of the wedge is flat and partially formed by three conserved tyrosines. The other surface is more rough and less hydrophilic in character. The importance of the highlighted residues in the CBD–cellulose interaction is discussed in the text.

between bound and free enzyme is described in the limit of low protein concentration by the following equation derived from a Langmuir adsorption model

$$[B] = [N_0]K_a[F]/(1+\alpha K_a[F]) \quad (1)$$

$$= K_r[F]/(1+\alpha K_a[F]) \quad (2)$$

where $[B]$ is the amount of bound protein on the cellulose surface (mol/g), $[F]$ is the concentration of free ligand (mol/liter), K_a is the association constant (liters/mol) and $K_r = [N_0]K_a$ is the relative association constant. α is a parameter showing the number of cellobiose units excluded from the cellulose surface by a single CBD molecule upon binding. $[N_0]$ is a constant describing the concentration of probable binding sites on a cellulose crystal surface (93 μmol lattice residues/g cellulose for the 1,1,0 face). Equation (1) was linearized to give

$$1/[B] = (1/K_a[N_0])(1/[F]) + \alpha/[N_0] \quad (3)$$

The slope and Y-axis intercept of a straight line fitted to Eq. (3) gave $K_r = K_a[N_0]$ and $\alpha/[N_0]$, respectively.

Electrostatic Calculations

The electrostatic CBD–CBD repulsion was estimated for hexagonally placed CBDs on the cellulose surface. From the CBHI binding data ($\alpha \approx 40$ mol/mol) the CBD–CBD distance was estimated to be 5.6 nm. This distance and the total CBD charge were used to obtain the CBD–CBD repulsion from the standard Coulomb expression, using a relative dielectric permittivity of 78 (water). The repulsion energy was then multiplied by six to account for the six closest neigbors.

RESULTS

Production of the CBHI CBD Mutant Proteins in *Trichoderma*

We have earlier produced three *T. reesei* CBHI CBD mutants, Y492A, Y492H, and P477R (Fig. 1), in the yeast *Saccharomyces cerevisiae*[13] and shown that all three mutations impair the functionality of the CBD. However, severe overglycosylation occurred in all forms of CBHI produced in yeast, and this interfered with their binding to cellulose. Therefore, for detailed characterization of the CBD mutants, the native host was used in this work for their expression.

Protoplasts of the engineered *T. reesei* strain VTT-D-93201 lacking the *cbh1* gene were transformed with plasmid pEM-F5 containing the 2400 bp *cbh1* promoter sequence, *cbh1* cDNA and 630 bp flanking 3′ sequence essentially as described elsewhere.[7] After screening and purification of the CBHI expressing clones, mutant proteins were produced in 2-liter shake flask cultivations yielding 200–300 mg of active CBHI protein per liter of culture supernatant. The molecular weights of purified mutant proteins

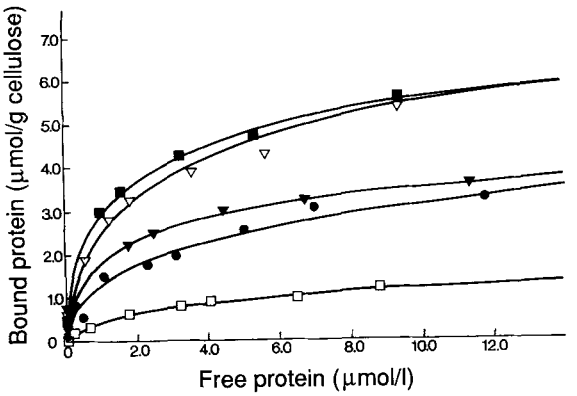

Fig. 2. Comparison of the binding isotherms of CBHI CBD mutants measured in 50 mM McIlvaine buffer, pH 5.0. Free and bound cellulase concentrations were determined after 90 min incubation at 4°C; 1 mg/ml bacterial cellulose was used as substrate. (■) Native CBHI, (□) CBHI core, (●) mutant Y492A, (▼) mutant Y492H, (▽) mutant P477R.

were identical to that of the native CBHI protein as judged by SDS electrophoresis (data not shown). The proteins were further analyzed by Western blotting with two monoclonal antibodies specific to the CBHI CBD (data not shown). The antibody H4 reacted with all of the mutant proteins whereas the antibody cI-89, recognizing the very "tip" of the CBD, i.e., the region around Y492,[23] reacted with all the mutants except Y492A.

Adsorption of Mutant Proteins to Bacterial Cellulose

The equilibrium binding isotherms of the wild type, the three mutants, and the catalytic domain of CBHI measured at pH 5.0 are presented in Figure 2. At the highest concentrations used, approximately 6 μmol/g of native CBHI protein was adsorbed. Binding of the core protein and of the two Y492 mutants were approximately 20 and 60% of that of the native CBHI, respectively. Contrary to our earlier results with yeast-produced proteins, the mutant P477R adsorbed to the same extent as the native CBHI.

The binding isotherms consisting of 15 data points were also measured at pH 2.2, 5.0, and 6.5 using McIlvaine phosphate-citrate buffers with constant ionic strength. The binding data ([Free] > 0.5 μM) is presented as histograms in Figure 3. Replicate independent experiments indicated an experimental uncertainty in the order of 10–15%. Thus the pH of the reaction mixture had a moderate but significant effect on cellulose binding. Moreover, the pH did not have any effect on protein concentration in the control experiments lacking cellulose, which indicates that the pH did not influence the stability of the proteins under conditions used. The pH dependences of the native CBHI, core protein, and mutant Y492A were similar, showing best binding at pH 5.0. The mutant P477R behaved similarly, except that the binding was better at pH 6.5 than at pH 2.2. The binding of mutant Y492H was distinctly decreased at acidic pH, and better at pH 6.5 than at pH 5.0.

The effect of ionic strength on the adsorption was examined by performing the adsorption experiments in water, in 1 M NaCl (pH 5.0) and in 1 M $MgSO_4$(pH 5.0) (Fig. 4). $MgSO_4$ (1 M) increased the adsorption of all proteins, whereas the same concentration of NaCl affected only the adsorption of native CBHI with intact CBD. The difference between native CBHI and the Y492 mutants was smaller in water than at high ionic strength. The mutant P477R adsorbed better than the native CBHI in water but less efficiently at high ionic strength.

The lower concentration data points were used to estimate the relative association constants, K_r, by an overlapping binding site model (as discussed below) (Table I). An example of the double-reciprocal plots used in the determination of the parameters for mutant P477R is shown in Figure 5. Comparison of the relative affinities in Table I shows that the core protein had the lowest affinity for cellulose and that the mutation Y492A reduced the apparent affinity of intact CBHI. A shift to more alkaline pH slightly increased the apparent affinity of all proteins except the CBHI core. Increasing the ionic strength led to the greatest improvement of the affinities of native CBHI and the core.

The overlapping binding site model also allowed approximate estimation of the parameter α, which basically represents the number of cellobiose units covered by a single protein molecule on the cellulose surface (see below). In addition to simple steric overlap, protein–protein interactions (repulsion or attraction) may influence the packing of the molecules even at moderate concentrations. Therefore the parameter α should rather be considered as a number of excluded cellobiose units from cellulose surface by a protein molecule. At pH 5.0 α was lowest with the native CBHI (40 mol/mol or 22 nm^2) and highest with the CBHI core (650 mol/mol or 350 nm^2).

Enzymatic Activity

The activity of CBHI on the small, soluble substrate methylumbelliferyl cellobioside was not affected by the mutations (data not shown). The activities on bacterial cellulose were calculated from the velocity of reducing sugar formation during short incubation times (30–120 min) (Table II). All the mutations reduced the activity of CBHI on insoluble cellulose. The mutant Y492H showed relatively higher activity at pH 6.5 than at pH 3.5. In high ionic strength (1 M $MgSO_4$), the activities of all the mutants were practically identical to that of the native CBHI (Table III), and the activity of CBHI core was increased from 20 to 50% of the activity of the wild-type enzyme.

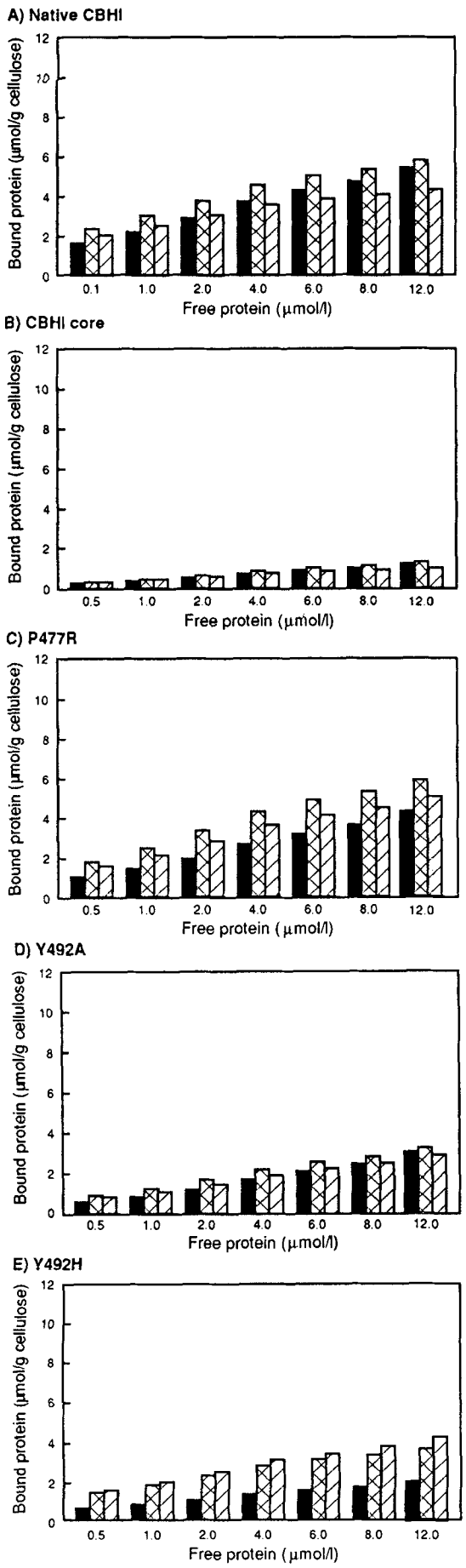

Fig. 3. (**A–E**) The effect of pH on binding of the CBHI CBD mutants. McIlvaine (citrate-phosphate) buffer was used in all experiments. The values have been derived from the binding isotherms consisting of 15 data points. (■) pH 2.2, (▨) pH 5.0, (▨) pH 6.5.

DISCUSSION

Comparison of the Different Forms of CBHI Expressed in *S. cerevisiae* and *T. reesei*

When CBHI, its core, and the three CBD mutants were expressed in *S. cerevisiae* the binding of intact CBHI and its core to cellulose was reduced by 30–45%, and the activity of CBHI on solid cellulose was reduced by half.[13] We also observed extensive overglycosylation and that the apparent molecular weight of intact CBHI was increased. The characteristics of the CBD mutant proteins expressed in the two organisms show clear differences, especially for the mutant P477R.

The structures of the glycans of CBHI expressed in *S. cerevisiae* were not determined, but their dimensions can be estimated. Digestion with endoglycosidase-H confirmed that most of the glycans present in the CBHI produced in yeast were N-linked oligosaccharides.[13] N-linked glycans are relatively short in *Trichoderma*[31] but in yeast consist of an extended mannose backbone with numerous large side chains.[32] It is known that the outer chain can be up to 100 residues long. A mannose unit is approximately 5 Å long, and the maximal chain length is approximately 500 Å. If the glycan is a random coil its end-to end distance would be $n^{0.5}L$ = 50 Å, where L is the monomer length and n is the degree of polymerization.[33] Since the rotation of the individual monomers is restricted, the actual extension is between 50 and 500 Å. From small angle X-ray scattering data the distance from the core domain to the CBD has been estimated to be ≈ 110 Å.[34] Therefore it is possible that the glycans attached to the N-glycosylation sites of the CBHI core[31] can interfere sterically with the binding of the CBD. In addition to steric hindrance, the yeast glycans are known to contain phosphate groups which can interact with charged groups at the CBD. The strong positive charge on the P477R CBD, in particular, may attract the phosphates of the oversized yeast glycans, which therefore mask the P477R CBD. This would explain why the P477R mutant produced in yeast bound much less efficiently than the same mutant produced in *T. reesei*. The reaction with monoclonal antibodies supports this hypothesis: the antibody H4, which did not recognize the mutant P477R expressed in yeast, reacted with the synthetic mutant CBD peptide and with mutant protein expressed in *Trichoderma*.

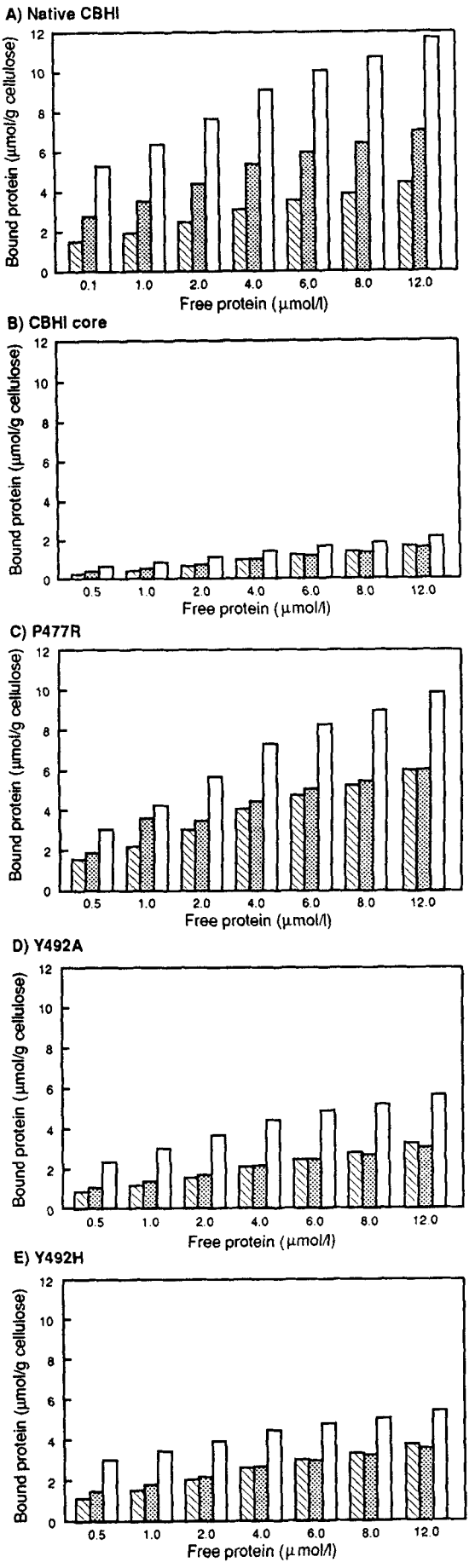

Fig. 4. (**A–E**) The effect of ionic strength on binding of the CBHI CBD mutants. The ionic strength was varied by adding 1 M $MgSO_4$ or 1 M NaCl to 50 mM McIlvaine buffer, pH 5.0. The values were derived from the binding isotherms. (□) water, (▨) NaCl, (▧) $MgSO_4$.

Apparent Affinities and Binding Capacities of CBHI and CBHI Core

Binding of cellulases on cellulose does not obey a simple one binding site model over a broad concentration range. In contrast to normal enzyme–substrate interaction, there are no definite binding sites on the cellulose surface, and the protein molecules can interact with each other during the adsorption process. Therefore, instead of applying classical multiple binding site models we used an "overlapping binding-site adsorption model"[28] to analyze the binding data. In this model, the cellulose surface is considered as a two-dimensional lattice of cellobiose units. The size of a cellulase molecule exceeds the size of one cellobiose unit and consequently the potential binding sites overlap. Thus the binding depends on the number of protein molecules bound and on their distribution on the cellulose surface. Considering the adsorption only at very low surface coverage simplifies the analysis. Under these conditions, one enzyme molecule does not prevent the binding of a second molecule. The apparent affinity, K_r, was obtained from the limiting slope of the adsorption data plotted in the form 1/[bound protein] as a function of 1/[free protein], emphasizing the lower concentration range. The value of K_r obtained is analogous to the partition coefficient used to estimate affinities of cellulases in many studies.[35,36] It is readily seen from the double-reciprocal plot in Figure 5 that the binding data obeyed the model only at low protein concentrations. Similar deviation from the model at high protein concentrations was also observed with *C. fimi* cellulases.[28]

At pH 5, the relative affinity K_r for native CBHI was 50 liters g^{-1}. Interestingly, this value was comparable to those obtained for *Cellulomonas fimi* CenA (40.5 liters g^{-1}) and Cex (33.3 liters g^{-1}).[28] The affinity of CBHI core was one order of magnitude weaker, indicating that the core contributes much less to the binding. With an $[N_0]$ of 93 μmol/g (see below), calculation of the molar affinity, K_a, gives 5×10^5 and 8×10^4 M^{-1} for native CBHI and core protein, respectively. All reported affinities for CBHI on bacterial cellulose are 100-fold lower than for Avicel at low protein concentrations.[37] This emphasizes the structural difference between bacterial cellulose and heterogenous semicrystalline Avicel isolated from wood fibers.

Saturation of the cellulose could not be attained despite rather high initial protein concentrations (15 μM/g/liter). The maximum amount of protein bound on the cellulose (B_{max}) was estimated to 6

TABLE I. Relative Affinity, K_r (liters/g), of CBHI CBD Mutants in Different Conditions*

Protein	Water	$MgSO_4$	NaCl	pH 2.2	pH 5.0	pH 6.5
Native CBHI	4	13	5	3	5	6
CBHI core	0.6	3	0.8	0.7	0.8	0.8
P477R	4	5	5	2	3	4
Y492A	2	6	3	0.8	1	2
Y492H	4	4	4	3	4	5

*The affinity was graphically estimated from the double reciprocal plots [Eq. (3)] of the adsorption data.

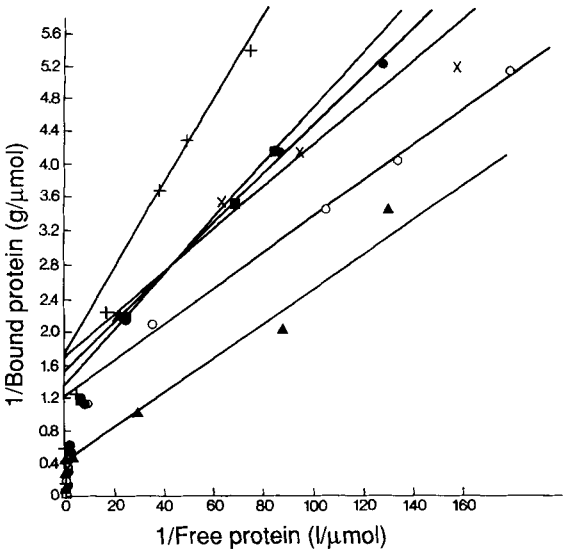

Fig. 5. An example of a double-reciprocal plot of the mutant P477R adsorption data. The relative affinity K_r in Table I was estimated from the limiting slopes of the plots according to Eq. (2) (see Materials and Methods). (+) pH 2.2, (■) pH 5.0, (●) pH 6.5, (×) water, (○) 1 M NaCl, (▲) 1 M $MgSO_4$.

μmol/g for intact CBHI, which was almost identical to that of *C. fimi* CenA (7 μmol/g). Contrary to bacterial cellulases, the core protein of CBHI clearly adsorbed to cellulose, although with 10-fold smaller affinity and 5-fold lower adsorption capacity. The binding of the isolated core has been demonstrated by many groups[21,37] and it conceivably indicates a difference in the adsorption mechanisms of fungal and bacterial cellulases. Whereas the *T. reesei* CBHI uses both the CBD and the catalytic domain for binding, the bacterial cellulases interact primarily by their CBD.[28]

Do CBHI and Its Core Have Different Preferred Binding Sites on the Cellulose Surface?

Bacterial cellulose is synthesized as a long ribbon composed of aggregated microfibrils, which is disrupted to individual microfibrils during the preparation of microcrystalline cellulose. On the basis of the three-dimensional structure[40] cellulose crystals have two nonidentical surfaces (Fig. 6) with Miller indices 1,1,0 and 1,−1,0.[39] Electron microscopy data from algal cellulose crystals suggest that CBHI adsorbs preferentially to their 1,1,0 face.[38]

On the basis of the density of Gardner and Blackwell[40] cellulose (1.63 g/cm^3) and the dimensions of a bacterial cellulose microfibril [40 nm (1,−1,0) by 15 nm (1,1,0)],[41,42] it can be estimated that the 1,1,0 face contains approximately 93 μmol cellobiose residues/g cellulose ($[N_0]$). Assuming that the molecules adsorb only on one crystal surface at low protein concentrations, the number of interacting cellobiose units, α, can be estimated from the double-reciprocal plot of the binding data by extrapolation [Eq. (3)].

The value of α obtained for native CBHI indicates that it occupies approximately 40 cellobiose units at pH 5.0. On the basis of the dimensions of their 3D structures,[4,43] the CBHI CBD is capable of interacting with approximately 10 cellobiose units, and its catalytic core with approximately 36–54 cellobiose units. Comparison of the experimental and calculated figures is thus consistent with simultaneous binding of both domains. However, the adsorption parameters for the isolated core domain indicate coverage of as many as 650 cellobiose units, which is about 14 times more than expected from the X-ray structure.

The CBHI core domain contains a 40-Å-long tunnel-shaped active site extending through the enzyme molecule.[43] It is therefore possible that the shape of the active site structurally restricts binding of the core to highly amorphous regions, loose polysaccharide chains, and particularly cellulose chain ends. Consequently, if the core binds poorly to the 1,1,0 surface, the $[N_0]$ value used to calculate the α value would no longer be valid. Recalculation [using Eq. (3)] of the $[N_0]$ for the core based on the surface coverage estimated from its X-ray structure (α = 45 mol/mol) gives a value of 6.4 μmol/g cellulose. Interestingly, with bacterial cellulose microfibril dimensions, this would correspond approximately to the area of either the two sharp or the two obtuse edges of a microcrystal. Recalculation of the $[N_0]$ would in fact also increase the molar affinity K_a for the core protein to its preferred binding sites ($K_a = K_r/[N_0]$).

TABLE II. Activities of the CBHI CBD Mutants on Bacterial Cellulose*

	Activity (mkat/gliter^{-1}/mol)				
pH	Native CBHI	CBHI core	P477R	Y492A	Y492H
2.2	0	0	0	0	0
3.5	62 ± 4	10 ± 0.4	46 ± 3	15 ± 1	18 ± 1
5.0	79 ± 7	8.2 ± 0.9	48 ± 2	18 ± 2	31 ± 0.5
6.5	32 ± 3	6.5 ± 0.6	20 ± 0.6	12 ± 0.4	25 ± 3
8.2	0	0	0	0	0

*Activities were calculated from the velocity of reducing sugar formation at 50°C. The errors were calculated as the standard deviation of several experiments.

TABLE III. The Effect of 1 M $MgSO_4$ on the Activity on Crystalline Cellulose*

	Cellobiose mM			
	No $MgSO_4$		1 M $MgSO_4$	
Protein	1 h	4 h	1 h	4 h
Native CBHI	0.73	1.2	0.61	1.0
CBHI core	0.14	0.31	0.30	0.46
P477R	0.65	1.1	0.62	1.2
Y492A	0.31	0.42	0.60	0.87
Y492H	0.40	1.0	0.60	1.2

*Cellobiose was quantified by HPLC after 1 and 4 h incubation with 2.1 μmol CBHI/g bacterial cellulose. The incubation temperature was 50°C.

Effects of the CBD Mutations on Binding and Activity

The apparent affinities of the wild-type and mutated CBHI were very similar. Only the core and the mutant Y492A had significantly reduced affinities (Table I). The mutations at the invariant Y492 reduced the binding capacities at pH 5.0 approximately by half. The effects of the mutations were moderate in comparison to the effects reported for similar mutations in bacterial CBDs.[14] However, the results were reproducible with separate enzyme batches. Moreover, it is known that CBHI preparations are very stable under the conditions used. Therefore, the differences are significant and not caused by variations in the quality of protein preparations.

The activities on cellulose correlated well with the binding properties of the CBD mutants. The mutant P477R with an intact hydrophilic surface on its CBD bound most efficiently (Table I, Figs. 2–4), and had the least reduced activity (Tables II and III). The affinity and the activity of the mutant Y492H were also less affected, in particular at pH 6.5, but the mutant Y492A had about 4- to 5-fold reduction in both properties. The core domain without a CBD bound least efficiently and had only 20% of the wild-type activity. Since neither the adsorption nor the activity of the mutant P477R was significantly altered it seems unlikely that the upper, more hydrophobic CBD surface is in direct contact with cellulose. These results confirm that adsorption via the CBD is required for efficient hydrolysis of crystalline cellulose, and support the hypothesis that productive primary cellulose–CBD interaction is transmitted through the flat hydrophilic CBD surface.

Effects of pH on Adsorption

Effects of pH on cellulase adsorption have been studied by many groups but with contradicting results.[36,44–46] We observed an approximately 2-fold decrease in the affinity at low protein concentration when the pH was decreased from 6.5 to 2.2 (Table I). Since the core affinity was insensitive to pH, the observed pH dependence of the native enzyme must be brought about by the CBD. There is only one ionizing amino acid side chain (H465) in the CBD of CBHI. The histidine residue H465 is stacked on top of Y466, which together with other conserved tyrosines (Y492 and Y493) and two invariant residues, N490 and Q495, is capable of forming hydrogen bonds with glucose hydroxyls or glycosidic oxygens. At low pH (<6.2), the H465 will become positively charged and preferably attract the hydroxyl oxygen (O_η) of the Y466 side chain. The reduction in affinity may therefore be caused by weakened hydrogen bonding with cellulose which is known to be important in protein–carbohydrate interactions.[10–12]

At high enzyme concentrations, the adsorption of CBHI is most probably affected by electrostatic repulsion between close-packed molecules. As can be seen in Figure 3, the adsorption of all proteins except the mutant Y492H was somewhat increased at pH 5. At this pH, closest to the isoelectric point (pI = 3.9), the overall charge of the molecules is lowest and electrostatic repulsion is minimal, which thus allows denser packing. The adsorption of the mutant Y492H was restored almost to the level of native CBHI at pH 6.5 but was drastically decreased at pH 2.2. This difference presumably reflects the ionization of the mutated CBD. Assuming the pK_a of a histidine to be 6.2, the tip histidine H492 and also H465 are totally protonated at pH 2.2, whereas approximately 50% of these residues are neutral at pH 6.5. At low pH, the carboxy terminal is also partially protonated, and the net charge approaches +2, leading to an electrostatic repulsion (≈5 kJ/mol at close packing) between the CBDs, which is four times

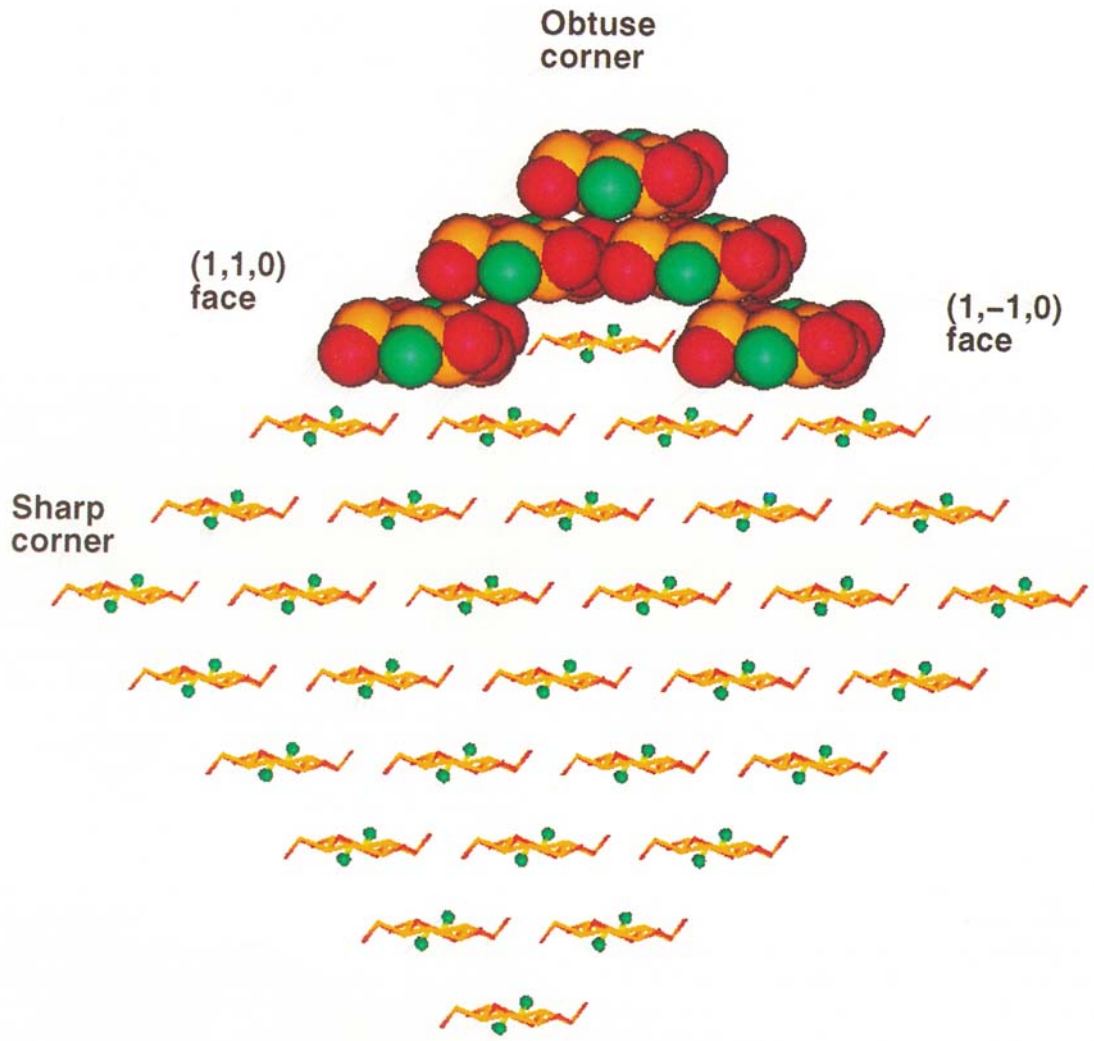

Fig. 6. The structure of crystalline cellulose I according to Gardner and Blackwell.[40] Microfibril cross section drawn from the nonreducing end toward the reducing end. Stick model with hydroxyl oxygens in red and glycosidic oxygens as small green balls. Five strands at the top obtuse corner have been drawn with van der Waals radii.

stronger than for monovalent domains at pH 6.5. Electrostatic repulsion may also explain the difference between the adsorption properties of the mutant Y492H produced in *Trichoderma* and in yeast. When the same protein was produced in yeast, the oversized, negatively charged glycans may have screened the charge of the H492, leading to almost wild type adsorption even at pH 5.[13]

Effect of High Ionic Strength

High concentrations of stabilizing salts such as NaCl and Na_2SO_4 have been found to increase and chaotropic salts to decrease the affinity of both bacterial and fungal cellulases to crystalline cellulose.[15,36] This is in agreement with the finding that denaturing agents are capable of eluting the cellulases bound on cellulose.[47,48] In this work, an increase in the ionic strength generally improved the apparent affinity and binding capacity of CBHI. These salt effects were moderate in 1 M NaCl but substantial in 1 M $MgSO_4$, with 4-fold higher ionic strength. The salt ions did not result in precipitation, denaturation, or "salting-out" of the proteins, because no reduction in the protein concentration was observed in the incubation controls which did not contain any cellulose.

It is unlikely that the different effects of NaCl and $MgSO_4$ would be caused by a specific cation effect involved in the interaction, because the CBHI CBD does not possess any charged residues nor a putative ion binding site. One possible explanation for the improved binding in the presence of salt ions is that they mask the electrostatic repulsion between protein molecules. The result also suggests that the in-

teraction between CBHI and cellulose may involve a hydrophobic effect.[49] The CBDs of fungal cellulases are in fact hydrophobic carrying relatively few ionizing amino acid side chains. It is very difficult to explain the hydrophobic effect in structural terms, because it is a sum of complex interactions. In addition to the direct cellulose–cellulase interaction, also water–water, water–cellulose, water–protein, and protein–protein interactions are involved. During binding, water molecules are displaced from the cellulose and CBD surfaces and the water structure around the molecules is changed. Generally, the main component in the hydrophobic effect is an entropic gain in water–water free energy when water is removed from the CBD and from cellulose surfaces that bind.

In spite of improved binding, the activities of the wild type CBHI and the mutant P477R were practically insensitive to high salt concentrations (Table III) but the activities of both of the Y492 mutants increased from 40 to 50% to almost full wild-type activity in 1 M $MgSO_4$. Apparently, the improved overall binding in high ionic strength compensates for the weaker binding of the mutated CBDs but further improvement of the wild-type binding no longer improves the activity. The enhancement of both the affinity (Table I) and the activity (Table III) of the CBHI core in high salt was particularly interesting since its binding capacity was not significantly affected (Fig. 4). Therefore, the increased activity of the core must be due to tighter binding to its preferred binding sites, not due to finding new productive sites. Once productively bound on the cellulose surface, the enzymes can solubilize cellulose with a mutated or with no CBD.

Mode of Binding and Activity of CBHI on Cellulose Crystals

The adsorption of cellulases on cellulose involves a number of weak interactions when different parts of the enzyme molecule bind to several adjacent glucose chains. As was shown in this study, an aromatic residue (Y492) on the hydrophilic surface of the CBD of CBHI is important in the binding. The interaction is partially formed by a ring current polarization attraction involving the delocalized π-electrons in the aromatic ring and the glucose pyranose ring. With a perfect cellulose crystal good stacking is possible only on the obtuse corners of the crystal with a small area of exposed 0,2,0 surface (Fig. 6). Therefore, the interaction is most probably formed also by hydrogen bonding between the enzyme and substrate. In all crystal surfaces, 1,1,0, 1,−1,0, and 0,2,0, enumeration of the functional groups shows that three hydroxyl groups (O_2, O_3, O_6) are available to form hydrogen bonds in each cellobiose unit (Fig. 6). The spacing and alignment of the three conserved tyrosines (Y466, Y492, Y493) and the residues N490 and Q495 are also perfect for achieving multiple interactions with the cellulose surface. Hydrogen bonding between the CBD and glucose chains may destabilize the intermolecular hydrogen bonds between the parallel glucose chains and together with water molecules release the chains from the crystal.

The glucose chain on the obtuse corner is the least stabilized by van der Waals contacts and remains without extensive intermolecular hydrogen bonding. This corner is probably the only productive binding site and the initial site of hydrolytic attack and at first the binding on the other surfaces would be nonproductive. When the first layer from the crystal corner becomes solvated, more 0,2,0 face is exposed and the molecules already bound on the other surfaces would consequently be relocated and bound on the next 0,2,0 surface perhaps due to the more favorable interactions with the tyrosines. Increase of the most accessible 0,2,0 surface would increase the number of interaction sites and result in tight adsorption on productive binding sites. The dissolution of the chains closest to the 1,1,0 and 1,−1,0 surfaces would be assisted by binding of the CBDs on the edge of the glucose layer next to the exposed 0,2,0 face on the top.

The bound CBDs may also prevent binding of the solvated chains back onto the crystal, thereby helping to feed individual glucose chains to the long active-site tunnel of CBHI. The active site contains seven binding sites for glucose and it is unlikely that, once bound, the polymeric glucose chain would dissociate from the enzyme. In this way the hydrolysis continues progressively along the chain. The processive mode of action also explains why CBHI is almost irreversibly bound to cellulose. Our data presented here and earlier[7] suggest that even though the core domain apparently needs chain ends for productive binding, the CBD leads the core to nonproductive binding sites along the cellulose crystals. Although this seems strange in terms of individual enzyme action, in nature, there are always endoglucanases present to create new chain ends for the bound exoglucanases to act upon.

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